94617

STIC-Biotech/Ch mLib

From: Hutzell, Paula
Sent: Wednesday, May 21, 2003 1:58 PM
To: Collins, Cynthia; STIC-Biotech/ChemLib
Subject: RE: rush sequence search request for SN 09/701926

approved

-----Original Message-----
From: Collins, Cynthia
Sent: Wednesday, May 21, 2003 12:53 PM
To: Hutzell, Paula
Subject: rush sequence search request for SN 09/701926

Paula,

Can you approve and forward this rush sequence search request? It is for an amended case.

Please search, **both** prior art and interference for SN 09/701926:

1) SEQ ID NO:1

Thank You,

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TYPE OF SEARCH:
NA Sequences:_____
AA Sequences:_____
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Questel/Orbit:_____
DRLink:_____
Lexis/Nexis:_____
Sequence Sys.:_____
WWW/Internet:_____
Other (specify):_____

09701926

TI Sequences affecting transposase-mediated silencing in plants and their use in regulating levels of gene expression and altering phenotypes

IN Carroll, Bernard John

PA The University of Queensland, Australia

SO PCT Int. Appl., 93 pp.
CODEN: PIXXD2

DT Patent

LA English

FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 9963068	A1	19991209	WO 1999-AU434	19990604

STIC-ILL

From: Collins, Cynthia
Sent: Wednesday, May 21, 2003 1:39 PM
To: STIC-ILL
Subject: ill order for 09/701926

ILL Ordering Information
Art Unit or Location < 1638>

Telephone Number <605-1210 >

Application Number or Other Order Identifier 09/701926

TI Characterization and expression of caffeoyl-coenzyme A 3-O-methyltransferase proposed for the induced resistance response of Vitis vinifera L.
AU Busam, G.; Junghanns, K.T.; Kneusel, R.E.; Kassemeyer, H.H.; Matern, U.
AV DNAL (450 P692)
SO Plant physiology, Nov 1997. Vol. 115, No. 3. p. 1039-1048
Publisher: Rockville, MD : American Society of Plant Physiologists, 1926-
CODEN: PLPHAY; ISSN: 0032-0889

TI DNA methylation and plant development.
AU Richards, Eric J.
CS Dep. Biology, Campus Box 1137, Washington Univ., One Brookings Drive, St. Louis, MO 63130 USA
SO Trends in Genetics, (1997) Vol. 13, No. 8, pp. 319-323.
ISSN: 0168-9525.

TI Expression of the potato leafroll virus ORF0 induces viral-disease-like symptoms in transgenic potato plants
AU van der Wilk, Frank; Houterman, Petra; Molthoff, Jos; Hans, Fabienne; Dekker, Ben; van den Heuvel, Johannes; Huttinga, Harm; Goldbach, Rob
CS DLO Res. Inst. Plant Protection (IPO-DLO), Wageningen, 6700 GW, Neth.
SO Molecular Plant-Microbe Interactions (1997), 10(2), 153-159
CODEN: MPMIEL; ISSN: 0894-0282

TI Developmental abnormalities and epimutations associated with DNA hypomethylation mutations
AU Kakutani, Tetsuji; Jeddeloh, Jeffrey A.; Flowers, Susan K.; Munakata, Kyoko; Richards, Eric J.
CS Dep. Biol., Washington Univ., St. Louis, MO, 63130, USA
SO Proceedings of the National Academy of Sciences of the United States of America (1996), 93(22), 12406-12411
CODEN: PNASA6; ISSN: 0027-8424

TI Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development.
AU Finnegan E J; Peacock W J; Dennis E S
CS Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, Australia.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1996 Aug 6) 93 (16) 8449-54.
Journal code: 7505876. ISSN: 0027-8424.

TI Demethylation-induced developmental pleiotropy in Arabidopsis.
CM Comment in: Science. 1996 Aug 2;273(5275):574-5
AU Ronemus M J; Galbiati M; Ticknor C; Chen J; Dellaporta S L
CS Department of Biology, Yale University, New Haven, CT 06520-8104, USA.
NC GM38148 (NIGMS)
SO SCIENCE, (1996 Aug 2) 273 (5275) 654-7.
Journal code: 0404511. ISSN: 0036-8075.

TI Demethylation-induced developmental pleiotropy in Arabidopsis.
CM Comment in: Science. 1996 Aug 2;273(5275):574-5
AU Ronemus M J; Galbiati M; Ticknor C; Chen J; Dellaporta S L
CS Department of Biology, Yale University, New Haven, CT 06520-8104, USA.

Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 8449–8454, August 1996
Developmental Biology

Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development

(hypomethylation/methyltransferase/flower development/homeotic transformations)

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Contributed by W. James Peacock, May 8, 1996

ABSTRACT ***Arabidopsis* plants transformed with an antisense construct of an *Arabidopsis* methyltransferase cDNA (*METI*) have reduced cytosine methylation in CG dinucleotides. Methylation levels in progeny of five independent transformants ranged from 10% to 100% of the wild type. Removal of the antisense construct by segregation in sexual crosses did not fully restore methylation patterns in the progeny, indicating that methylation patterns are subject to meiotic inheritance in *Arabidopsis*. Plants with decreased methylation displayed a number of phenotypic and developmental abnormalities, including reduced apical dominance, smaller plant size, altered leaf size and shape, decreased fertility, and altered flowering time. Floral organs showed homeotic transformations that were associated with ectopic expression of the floral homeotic genes *AGAMOUS* and *APETALA3* in leaf tissue. These observations suggest that DNA methylation plays an important role in regulating many developmental pathways in plants and that the developmental abnormalities seen in the methyltransferase antisense plants may be due to dysregulation of gene expression.**

Methylation of cytosine residues, the most common modification of DNA in higher eukaryotes, has been implicated in gene regulation, genomic imprinting, the timing of DNA replication, determination of chromatin structure, and as a basis for epigenetic phenomena (for reviews, see ref 1). A fundamental role for DNA methylation in mouse development was demonstrated by establishing a targeted mutation of the DNA methyltransferase gene in the germ line of mice (2). Methylation levels in mouse embryos homozygous for the mutation were reduced to ≈30% that of heterozygous or wild-type mice and the homozygous embryos spontaneously aborted in midgestation. The underlying cause for the embryo lethal phenotype has not been determined, but the decreased methylation did result in abnormal expression of imprinted genes and *Xist* (3, 4).

In the plant *Arabidopsis thaliana* mutants with decreased DNA methylation (*ddm*) (5) developed morphological abnormalities after several generations of self-fertilization (6). These plants had rounded rosette leaves, an increased number of cauline leaves, and were late-flowering. These observations suggest that hypomethylation of plant DNA can lead to abnormal development. Cytosine methylation was reduced to ≈30% of wild type in plants homozygous for a mutation at the *ddm1* locus, and methylation of cytosines in both CG and CNG motifs was reduced (5). The biochemical basis for the reduction in methylation has not been determined but DNA methyltransferase activity was comparable to wild type (6), indicating that a methyltransferase gene or genes were not affected.

The isolation of a cDNA encoding a putative DNA methyltransferase (*METI*) of *Arabidopsis* (7) has enabled us to investigate the role of DNA methylation in plant development using a reverse genetics approach. We have directly targeted methyltransferase activity by introducing an antisense construct of this gene into wild-type *Arabidopsis*. Transgenic plants showed a reduction of up to 90% in cytosine methylation, predominantly in CG dinucleotides, suggesting that *METI* encodes a functional methyltransferase. Reduction of methylation resulted in dramatic changes in plant morphology, including homeotic transformations of floral organs. Plants with reduced methylation showed aberrant expression of floral homeotic genes; these genes, which are normally expressed only in floral tissue (8, 9), were also transcribed in leaves of transgenic antisense plants.

MATERIALS AND METHODS

Generation of the Methyltransferase Antisense Construct. The plasmid pYc8 (7) was cut with *Eco*RI and a 2.8-kb fragment of the methyltransferase cDNA was inserted between the cauliflower mosaic virus 35S promoter (10) and the nopaline synthase 3′ termination signal (11). The resulting plasmid was linearized with *Sal*I, which cleaves 5′ to the 35S promoter, and inserted into the unique *Sal*I site in the binary vector pBin19 (12), then mobilized into *Agrobacterium tumefaciens* strain AGL1 (13), using pRK2013 as a helper strain in a triparental mating.

Transformation of *Arabidopsis*. Transgenic *Arabidopsis* plants, ecotype C24, were obtained from root explants using the method described in Dolferus *et al.* (14).

DNA Isolation and Southern Hybridization. DNA was isolated from above-ground tissue, and Southern hybridizations were done according to the methods published in Taylor *et al.* (15).

Thin Layer Chromatography Assay. In this assay (5, 16, 17), DNA (1 μg) was cleaved with *Taq*I, which recognizes the sequence TCGA and cleaves 5′ to the cytosine, whether or not the cytosine is methylated. Cleavage with *Taq*I results in fragments of <500 bp, on average. The methylation status of the terminal cytosines was determined by labeling with [γ-^{32}P]ATP. Enzymatic digestion cleaved DNA to deoxynucleotide monophosphates, which were separated by thin layer chromatography (16). Radioactivity in individual deoxynucleotide monophosphates was quantitated using a Molecular Dynamics PhosphorImager and IMAGEQUANT software. Uncut DNA, for each sample, was treated in parallel to determine background radioactivity incorporated into deoxy-5-methylcytosine monophosphate (d^{m}CMP) and deoxycytosine monophosphate (dCMP) due to the presence of sheared DNA; this background was subtracted before calculating 5-methylcytosine levels, by the formula d^{m}CMP/d^{m}CMP + dCMP, which were then normalized to wild type.

Root Development. Surface-sterilized seeds from C24 and T_3 line 10.5 were placed on Murashige–Skoog medium solidified with 1.5% agar (18), ≈1 cm from one edge of 10-cm square culture plates. The sealed plates were placed vertically in a

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*To whom reprint requests should be addressed.

Table 1. Methylation levels in T_2 plants from five independent methyltransferase antisense families

Plant identification (n)	Copies of T-DNA*	Methylation level,† %
Family 4 (1)	3	82
Family 8 (2)	1	100.5 ± 3.4
Family 10 (3)	3	17.9 ± 6.5
Family 11 (2)	1	89.3 ± 7.3
Family 22-6 (2)	4	77.0 ± 0.83

All plants tested were from the T_2 generation; n, number of plants sampled.
*Number of copies of T-DNA inserted into the genome, as determined by number of T-DNA left border fragments
†% wild-type methylation ± SE.

controlled environment at 22°C under long days (16 hr fluorescent light, 8 hr dark). After 4 weeks, the length of the primary root and the number of roots initiating at the root–hypocotyl junction were determined.

Detection of the Transgene by PCR. The presence of the antisense transgene was detected using a polymerase chain reaction (PCR) assay for the selectable *Npt*II marker that was cotransformed on the same T-DNA. DNA was prepared from a single leaf from individual plants (19). DNA template (1 μl) was added to a reaction mix (20 μl) containing 10 mM Tris (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM primer 1 (GAGGCTATTCGGCTATGA), 1 μM primer 2 (ACTTCGCCCAATAGCAG), and 1 unit *Taq* DNA polymerase (Perkin–Elmer). Cycling conditions were 94°C for 4 min, 50°C for 30 sec, 72°C for 30 sec (one cycle), and then 34 cycles of 94°C for 10 sec, 50°C for 10 sec, and 72°C for 30 sec. The reaction products were resolved by electrophoresis on a 2% agarose gel—the expected size of the amplified fragment is 238 bp.

RNA Isolation and Northern Analyses. RNA was isolated from leaf tissue of plants grown in sterile culture or from plants grown in soil in controlled environment cabinets as described (14). Northern hybridizations were performed according to Dolferus *et al.* (14).

RESULTS

Arabidopsis (ecotype C24) was transformed with a *METI* antisense construct encoding the putative methyltransferase domain (520 aa) and ≈330 aa of the amino terminal domain (7) under the control of a constitutive promoter (10). Fifty independent transgenic (T_0) plants were regenerated. Progeny from a random subset of five of these plants were used for this study in which we measured the level of DNA methylation and monitored growth and development of plants in the T_2 and subsequent generations.

Table 2. Methylation levels in T_3 lines for methyltransferase antisense family 10

Plant identification* (n)	Transgene status†	Methylation level,‡ %
10.1 (1)	Homozygous	23.9§
10.1 (3)	Hemizygous	20.8 ± 1.3
10.1 (4)	Null	42.8 ± 1.8
10.2 (1)	Hemizygous	29.7 ± 2.0
10.2 (2)	Null	64.0 ± 4.1
10.5 (6)	Homozygous	13.3 ± 1.8
10.13 (4)	Homozygous	9.3 ± 0.83

n, number of plants sampled.
*T_3 progeny of individual T_2 plants, 10.1, 10.2, etc., from family 10.
†Transgene status was determined by testing for presence of the transgene in 24 progeny by PCR amplification of a fragment of the *Npt*II selectable marker. Data for individual plants with same transgene status, within each line, were pooled.
‡% wild-type methylation ± SE.
§Value for single plant, measured in duplicate and averaged.

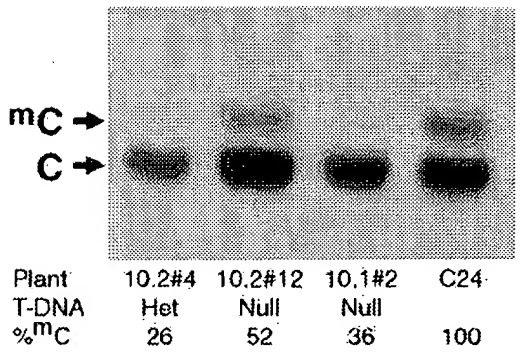

FIG. 1. Quantitation of DNA methylation by thin layer chromatography. 5-Methylcytosine levels, in a subset of CG dinucleotides, were estimated by quantitation of radioactivity, using a Molecular Dynamics PhosphorImager, then calculated by the formula $d^mCMP/(d^mCMP + dCMP)$ and then normalized to wild-type levels.

***METI* Antisense Reduces DNA Methylation.** The level of cytosine methylation in DNA from antisense plants was compared with control C24 plants in a thin layer chromatography assay (5, 16), which measures methylation in the subset of CG dinucleotides that occur in *Taq*I sites. Methylation in the five antisense transgenic families ranged from ≈10% to 100% of wild type (Tables 1 and 2). Family 10, with the lowest level of cytosine methylation, has three copies of the transgene, inherited as a single locus; family 4 and family 22–6, which had 70–80% of normal methylation, contained three and four copies of the transgene, respectively. Family 8 and family 11, in which methylation was unchanged, contained only one copy of the antisense transgene.

T_2 and T_3 antisense plants from family 10 had 10–30% of wild-type methylation (Fig. 1; Tables 1 and 2). Methylation levels varied between progeny of sibling T_2 plants and within T_3 lines (Table 2). In general, plants homozygous for the transgene construct had the lowest levels of DNA methylation (Table 2), suggesting that methyltransferase activity was inversely correlated with antisense expression. Methylation levels in homozygous plants of line 10.5 remained the same over three generations (Table 3).

T_3 plants from family 10 that did not inherit the antisense construct also showed reduced levels of DNA methylation relative to wild type (Table 2, plants 10.1 null and 10.2 null), although the methylation level, at 40–65% of normal, was higher than in hemizygous sibs (20–30% of wild type). When these antisense-null plants were selfed, the methylation levels in progeny were still lower than normal.

Methylation in plant DNA occurs predominantly in cytosines located in either CG dinucleotides or CNG trinucleotides (20, 21). We determined the specificity of *METI* antisense demethylation in centromeric and ribosomal DNA sequences. In wild-type *Arabidopsis*, the centromeric repeat is heavily methylated (20) and is not cleaved by *Hpa*II (Fig. 2*A*). In DNA from the antisense plants, this sequence was cleaved

Table 3. Methylation levels in three successive generations of plants in line 10.5 homozygous for the transgene

Plant identification* (n)	Methylation level,† %
T_2 10.5 (1)	14.8
T_3 10.5 (6)	13.3 ± 1.8
T_4 10.5 (6)	9.6 ± 0.5

n, number of plants sampled.
*Homozygous plants from different generations of line 10.5.
†% wild-type methylation ± SE.

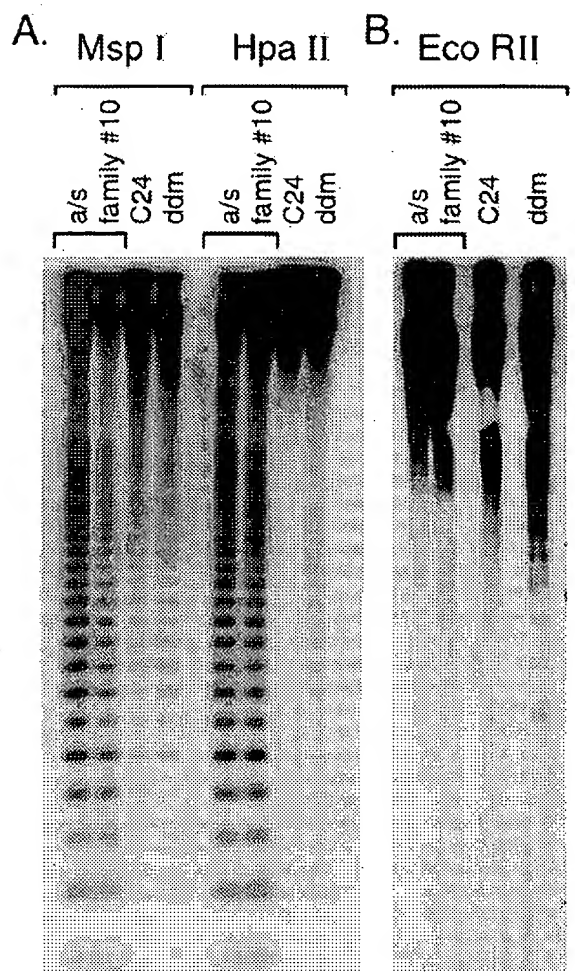

FIG. 2. Specificity of demethylation caused by *METI* antisense. (*A*) Southern analysis of DNA from individual T_3 10 methyltransferase antisense plants, homozygous *ddm1* (5), and untransformed C24, to detect changes in CG methylation. DNA was digested with either *Msp*I or *Hpa*II, which cleave the site CCGG but which differ in their sensitivity to DNA methylation. *Hpa*II will cleave this sequence only if both cytosines are unmethylated (23, 24), while *Msp*I will not cleave when the external cytosine is methylated (23, 24) but is only affected by methylation of the internal cytosine in some sequence contexts (25–27). A 180-bp repeat unit from the centromeric DNA of *Arabidopsis* was used as a probe (22). (B) Southern analysis of DNA from individual T_3 10 methyltransferase antisense plants, homozygous *ddm1* (5), and untransformed C24 to monitor changes in CA/TG methylation. DNA was cleaved with *Eco*RII, which cleaves the sequence CCA/TGG when the internal cytosine is not methylated (23). The Southern blot was probed with a fragment from the 5S ribosomal repeat of *Arabidopsis* (28). The apparent gap in hybridization to C24 DNA is due to incomplete DNA transfer.

by methylation sensitive *Hpa*II (Fig. 2*A*), indicating that the centromeric DNA was hypomethylated in antisense plants relative to wild type. In DNA from plants homozygous for the *ddm1* mutation (5), this sequence was also hypomethylated (Fig. 2*A*), but the pattern of demethylation differed between the antisense and *ddm1* plants. Cleavage of CG sites (tested with *Hpa*II) was greater in DNA from the antisense plants (Fig. 2*A*), whereas CAG and CTG sites (monitored with *Eco*RII) were cleaved more extensively in DNA from *ddm1* plants (Fig. 2*B*). These data suggest that the *METI* antisense preferentially reduces methylation in CG dinucleotides, whereas *ddm1* affects methylation of both CG and CNG (5).

*Msp*I cleaved centromeric DNA from both *METI* antisense and *ddm1* plants more extensively than DNA isolated from untransformed C24 (Fig. 2*A*), showing that methylation of the 5′ cytosine in the sequence CCGG was also reduced in the antisense plants compared with wild type. Thus, *METI* antisense may reduce activity of an enzyme that methylates both ^{m}CG and ^{m}CCG, like the partially purified CG methyltransferase from pea (29), or the *METI* antisense may interact with transcripts from two members of the methyltransferase gene family (7). A third possibility is that, in the antisense plants, demethylation of the internal cytosine, in that sequence context, affected *Msp*I cleavage (25–27).

The 17S ribosomal repeat sequence (5) was also cleaved more extensively by *Hpa*II in antisense plants than in C24. Families 4, 10, and 22–6, had reduced CG methylation in these repeat sequences.

Reduced DNA Methylation is Associated with Developmental Abnormalities. Morphological abnormalities in both vegetative and reproductive structures were observed in plants from the T_2 and subsequent generations. The abnormal plants had decreased stature, smaller rounded leaves, leaves with margins curled toward the upper leaf surface, decreased fertility, and reduced apical dominance resulting in a bushy appearance. Antisense plants had shorter roots (5.83 ± 0.21 cm, antisense; 7.3 ± 0.25 cm, wild type) with more branching at the root crown (1.91 ± 0.133, antisense; 1.34 ± 0.07, wild type). Phenotypes were variable with individual plants displaying some or all of these characteristics (Fig. 3 *A* and *B*). The severity of the abnormal phenotype correlated with the extent of demethylation. In families 4 and 22–6, which had a smaller reduction in methylation (Table 1), phenotypes were similar, but less severe (Fig. 3*B*), than those in family #10 (Fig. 3*A*).

Arabidopsis flowers have four different organs arranged in concentric rings or whorls; there are four sepals in the outer whorl, four petals in the second whorl, six stamens (the male reproductive organs) in the third whorl, and, in whorl four, a single, female reproductive organ consisting of two fused carpels. Floral homeotic genes specify organ identity and their function is restricted to defined domains on the floral bud that are coincident with the organ whorls (for reviews, see refs. 8 and 9). Some flowers on the antisense transgenic plants showed homeotic transformations of floral organs (Fig. 3 *C–I*), with the flowers resembling those described on plants of floral homeotic mutants (9). We observed flowers with an increased number of stamens and reduced carpel tissue (Fig. 3 *C* and *I*), as in the *superman* (*sup*) mutant (31, 32); however, when carpels developed, they contained ovules of normal morphology. When organs in the inner two whorls were transformed into petals or staminoid petals (Fig. 3*E*), organ number increased (like *superman agamous* mutants; refs. 31 and 32). In other flowers, where sepals were replaced by carpelloid tissue, the number of organs in whorls two and three decreased (Fig. 3 *F* and *H*) as in *apetala2* mutants (33). Sometimes extra flowers developed in place of a floral organ or in the internode between floral organs (Fig. 3*G*), comparable to mutation in *apetala1* (34).

Flowers on a single plant showed a spectrum of these phenotypic abnormalities and flowers formed later in development were more severely affected (Fig. 3*D*). Floral abnormalities were most common and diverse in family 10, the family with the lowest level of DNA methylation. Abnormal flowers were observed in all antisense families, with reduced methylation, and similar phenotypes were observed on plants with equivalent methylation levels (compare Fig. 3 *F* and *H*). This, together with the observation that, in family 10, some antisense-null plants with 40–65% of normal methylation produced abnormal flowers (Fig. 3*F*), suggests that the floral abnormalities arose as a result of decreased DNA methylation rather than by mutation at the site of the T-DNA insertion.

The hemizygous T_1 10 plant, which had reduced apical dominance and fertility, was phenotypically different from the T_1 plants in the other families used in this study. Plant and

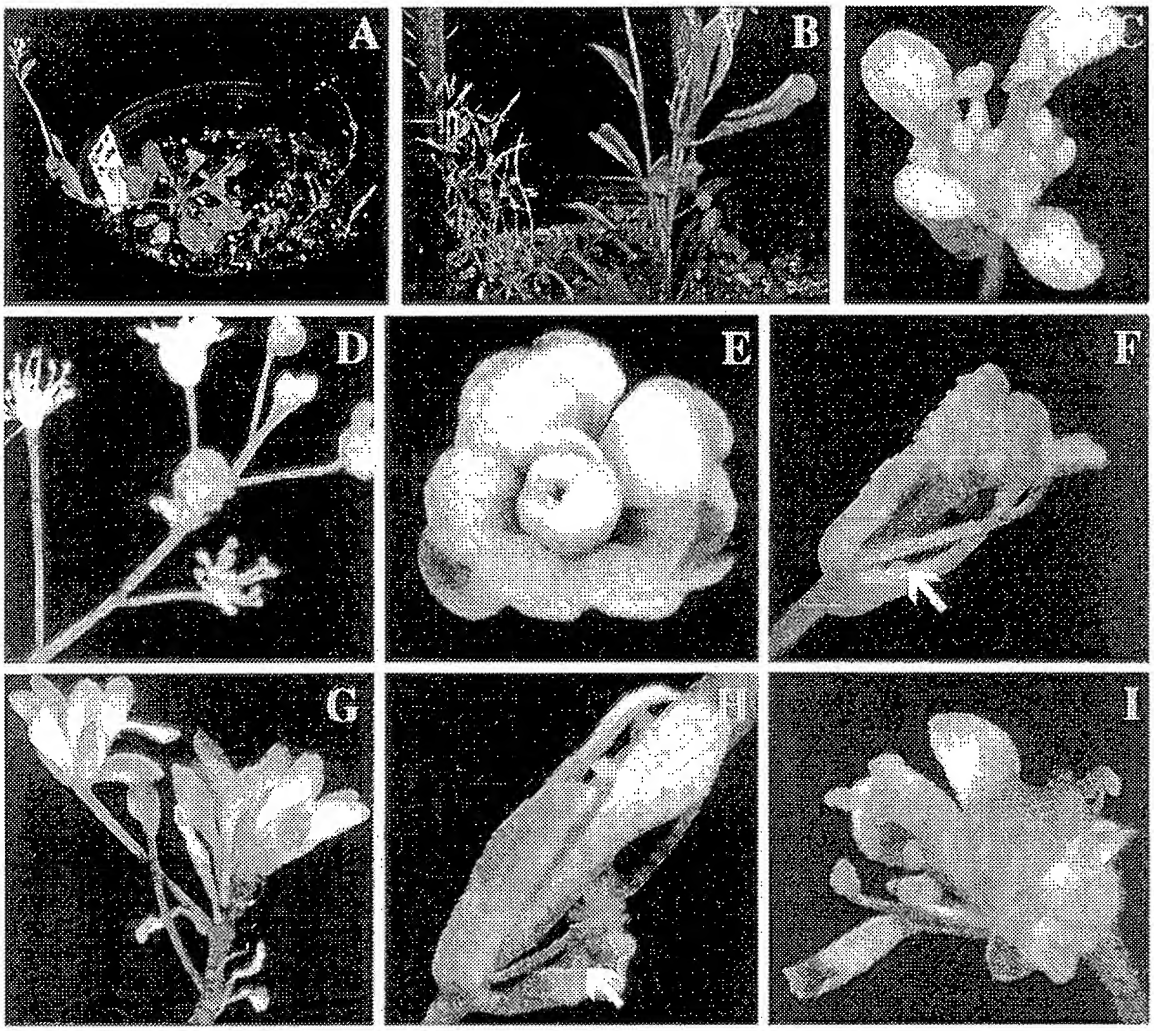

FIG. 3. Phenotypic abnormalities in plants and flowers from methyltransferase antisense plants with reduced levels of DNA methylation. (*A*) Flowering T_2 plants from family 10, both of which carry the antisense construct. The plant on the left has a relatively normal phenotype whereas the plant on the right has decreased apical dominance (is branched), smaller leaves and is greatly reduced in size (reproduced from ref. 30). (*B*) Sibling T_2 plants from family 22–6, both of which carry the methyltransferase antisense. The plant on the left is smaller and bushier than normal, while the plant on the right has leaves which curl to the upper surface. (*C*) A flower from a hemizygous T_3 plant, line 10.1. The organs in the outer two whorls are normal but there are 12 stamens and the carpels of gynoecium (female reproductive organ) are unfused. (*D*) An inflorescence from a homozygous T_4 plant, line 10.5. The early flowers which have extra stamens were followed by flowers with four sepals then petals or staminoid petals in all internal whorls. (*E*) A flower from a hemizygous T_3 plant, line 10.2. This flower has an increased number of organs with four normal sepals then petals or staminoid petals in all the inner whorls. (*F*) A flower from an antisense-null T_4 plant, line 10.2. This flower has no petals and a reduced number of stamens in the third whorl. Two sepals are carpelloid with stigmatic papillae and has ovules on the margin (indicated by an arrow). (*G*) A flower from a homozygous T_4 plant, line 10.5. This flower has an increased number of whorls (note the dried petals, extending down the stem), all organs internal to sepals are petals or staminoid petals, and there are secondary flowers within this flower. (*H*) A flower from a T_3 plant, line 4.1. This flower has a carpelloid sepal with ovules on the margin (indicated by an arrow) and a reduction in the number of organs in whorls two and three. (*I*) A flower from a T_2 plant, line 39.25. This flower has normal organs in the outer two whorls but it has 12 stamens and the carpels of the gynoecium are not fused.

floral phenotypes in family 10 became more abnormal in successive generations. The homozygous T_2 plant, 10.5, was reduced in stature but otherwise normal, whereas all its T_3 progeny had aberrant flowers that were usually sterile. The few T_4 progeny had additional floral homeotic transformations, and most were completely infertile.

A sixth family, 39, had methylation levels (10–20% of wild type) comparable to family 10. The T_1 plant in this family had reduced apical dominance, decreased fertility, and some abnormal flowers. Most flowers on its T_2 progeny were abnormal, and the floral phenotypes (Fig. 3*I*) were similar to those in family 10 (Fig. 3*C*). The types of floral abnormalities correlate with the extent of demethylation, suggesting that the homeotic transformations were caused by the same mechanism in both families.

In addition to the morphological abnormalities observed in the antisense plants the timing of the transition from the vegetative to the reproductive phase of development was altered (data not shown), indicating that DNA methylation is involved in the timing of developmental processes.

Reduced DNA Methylation Is Associated with Aberrant Gene Expression. The floral homeotic transformations that we observed in methyltransferase antisense plants suggested that the expression of floral genes may be abnormal. We found that the floral homeotic genes, *AGAMOUS* (*AG*) and *APETALA3* (*AP3*), were expressed in leaves of methyltransferase antisense plants, whereas in wild-type plants, expression of these genes is confined to restricted domains of the floral bud (8, 9). Fig. 4 shows that *AP3* transcripts were detected in leaf RNA from antisense plants from families 22–6, 10, and 39, but not in

untransformed C24. Similar results were obtained for *AG*, indicating that activation of transcription in vegetative tissue was not unique to *AP3*. Demethylation of genomic DNA resulted in ectopic expression of these genes in the antisense plants.

DISCUSSION

Methylation Patterns Were Not Restored in Each Generation. The introduction of the *METI* antisense construct resulted in reduced levels of DNA methylation in the progeny of about 60% of the primary transformants. Methylation levels varied both between and within antisense families. The variation seen within a family reflected a difference in copy number of the transgene; plants that were homozygous for the antisense showed the greatest demethylation. In some progeny that did not inherit the transgene, methylation levels remained below wild type, suggesting that normal methylation levels were not restored in a single generation. Vongs *et al.* (5) found in progeny of a cross between a *ddm1* homozygote and a wild-type plant that, just as in the antisense-null plants, *de novo* methylation was slow, if indeed it occurred at all. Repeated crossing to wild-type plants gradually restored the methylation level; this is consistent with progressive loss of hypomethylated DNA, rather than *de novo* methylation (5). These data indicate that *Arabidopsis* plants lack mechanisms to restore the normal level of methylation after passage through meiosis, suggesting that plants may not undergo the cycle of general demethylation followed by remethylation, observed in early stages of mammalian embryo development (for review, see ref. 36).

DNA Methylation Plays a Role in Plant Development. Plants with low levels of DNA methylation displayed many phenotypic abnormalities suggesting that leaf, root, inflorescence, and flower development were perturbed. Phenotypes were variable between plants of the same family and became more severe in successive generations, even though methylation levels remained unchanged. Homozygous *ddm1* plants also showed phenotypic abnormalities after several generations of selfing (6). The progressive increase in the phenotypic aberrations could result from the segregation of different demethylated sites among sexual progeny.

FIG. 4. Northern analysis showing expression of *AP3* in vegetative tissue of methyltransferase antisense plants. Total RNA isolated from leaves of pooled T_2 or T_3 progeny of antisense plants, or untransformed C24 plants grown in sterile culture. The blot was probed with an *AP3* (35) antisense transcript.

Developmental Abnormalities Correlated with Altered Patterns of Gene Expression. Our data suggest that ectopic expression of the two floral homeotic genes, *AP3* and *AG*, causes some of the floral homeotic transformations on plants with reduced levels of methylation. Other experiments have shown that ectopic expression of these two genes resulted in similar homeotic transformations; constitutively expressed *AP3* caused *sup*-like phenotypes and *apetala2*-like flowers resulted from constitutive expression of *AG* (37, 38). The *sup*-like flowers on the antisense plants had extra stamens but ovules were normal, similarly ovule development was unaffected in 35S-*AP3* transgenic plants (E.J.F., unpublished data); in contrast, mutation of the *SUP* gene increased stamen number and affected ovule development (31, 32, 39). These data suggest that SUPERMAN was functional in the *sup*-like flowers on the antisense plants and that the phenotype was a result of ectopic expression of *AP3*.

The type of floral abnormality was correlated with the extent of demethylation; *ap2*-like phenotypes occurred in plants with a small reduction in methylation whereas flowers on plants with methylation <35% of the wild-type level were *sup*-like. Severity of the floral phenotype increased during development; *sup*-like flowers were replaced, later in development, by flowers similar to the double mutants, *superman agamous*, *superman apetala1*, and, rarely, *superman apetala2*, as well as the *superman agamous apetala1* triple mutant (31, 32). In floral homeotic mutants, the gradient in severity of the floral phenotype is reversed, with early flowers being more severely affected than the later flowers (31, 34). The phenotypic gradients may result from increasing activity of other, redundant floral genes partially compensating for the mutation (8); increased activity of redundant genes could enhance the effects of ectopic gene expression in the antisense plants, resulting in the gradient of increasing severity seen in these plants. The more extreme phenotypes of later generations may be a consequence of the accumulation of different demethylated sites in sexual progeny.

Reducing DNA Methylation May Alter Gene Expression by Changing Chromatin Structure. Our observations suggest that the primary effect of the methyltransferase antisense transgene is to reduce DNA methylation. The methylation status of a gene, particularly of the promoter, can influence transcription (for review, see refs. 40 and 41). Ectopic expression could result directly from demethylation of promoter elements of the floral genes or of transcription factors that regulate them, or from an alteration in the chromatin structure surrounding these genes. Alternatively, gene expression could be altered by insertional mutagenesis caused by transposition of previously methylated, inactive transposable elements (for review, see ref. 41).

The phenotype of the *Arabidopsis curly leaf* (*clf*) mutant, which includes curled leaves, early flowering, and *ap2*-like homeotic transformation in late flowers (P. Puangsomlee and J. Goodrich, personal communication), is similar to that of some methyltransferase antisense plants. In both *clf* mutants and methyltransferase antisense plants the floral homeotic genes, *AG* and *AP3*, are expressed ectopically. The *CLF* gene encodes a protein with homology to a member of the *Drosophila* polycomb-group proteins indicating that chromatin structure is important in regulating plant gene expression. The *Drosophila* polycomb (Pc-G) and trithorax (trx-G) group proteins affect higher order chromatin structure, and polycomb group proteins are involved in long-term gene repression by formation of stable chromatin complexes (42).

The similarity of phenotypic abnormalities in *clf* mutants and methyltransferase antisense plants suggests that DNA methylation may act in concert with a Pc-G/trx-G-like system to stabilize determined states of gene expression in *Arabidopsis*. A link between DNA methylation and gene regulation by trx-G proteins has been proposed (43).

The fundamental importance of DNA methylation for normal development in the mouse has already been established, and, in other mammals, methylation has been implicated in the control of gene expression and imprinting. In plants, decreased DNA methylation has been correlated with promotion of flowering in *Arabidopsis* (44), but its role in regulating gene expression during development has not been demonstrated. The developmental abnormalities and aberrant patterns of gene expression observed in the transgenic plants, described here, demonstrate a role for DNA methylation in many pathways of plant development.

The authors wish to thank Eric Richards for providing seeds of *ddm1*, Justin Goodrich for seeds of *clf*, Justin Goodrich and Preeya Puangsomlee for communication of results before publication, Lyndall Thorpe for providing excellent technical assistance throughout this work, Stuart Craig for photography of the abnormal flowers, Tim Bestor for many stimulating discussions, and Mick Graham for critical reading of the manuscript. This work was supported in part by the "May Statement Initiative" funding for "Geneshears."

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TI Characterization and expression of caffeoyl-coenzyme A 3-O-methyltransferase proposed for the induced resistance response of Vitis vinifera L.
AU Busam, G.; Junghanns, K.T.; Kneusel, R.E.; Kassemeyer, H.H.; Matern, U.
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Publisher: Rockville, MD : American Society of Plant Physiologists, 1926-
CODEN: PLPHAY; ISSN: 0032-0889

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SO Trends in Genetics, (1997) Vol. 13, No. 8, pp. 319-323.
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CS DLO Res. Inst. Plant Protection (IPO-DLO), Wageningen, 6700 GW, Neth.
SO Molecular Plant-Microbe Interactions (1997), 10(2), 153-159
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SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1996 Aug 6) 93 (16) 8449-54.
Journal code: 7505876. ISSN: 0027-8424.

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AU Ronemus M J; Galbiati M; Ticknor C; Chen J; Dellaporta S L
CS Department of Biology, Yale University, New Haven, CT 06520-8104, USA.
NC GM38148 (NIGMS)
SO SCIENCE, (1996 Aug 2) 273 (5275) 654-7.
Journal code: 0404511. ISSN: 0036-8075.

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Chemical Shackles for Genes?

In both plants and animals, methyl group addition to DNA is proving to be necessary for normal development, apparently because it helps shut down genes

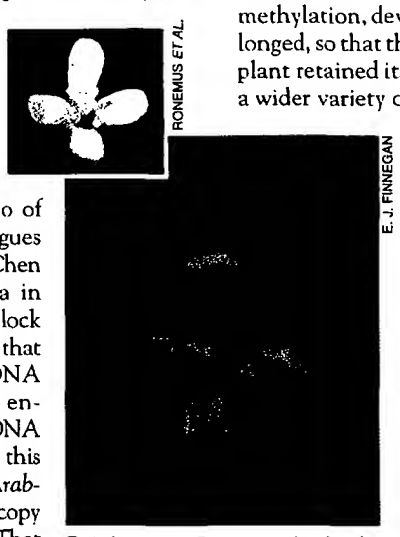
The more researchers learn about the way genomes work, the more genes seem like books in a library: Just a few are read at any one time, while the rest gather dust. Still unclear, however, is what kind of "librarian" runs the genetic library. Does the librarian actively pick out the right genes to be read, or does it maintain order by restricting the reading of all but a key few? Now, after decades of debate, researchers are converging on at least part of the answer.

In studies performed on the small plant *Arabidopsis thaliana*, three separate groups, one of which reports its results on page 654, have found evidence that the librarian does its job by placing certain genes off-limits. When the researchers genetically modified the plants to reduce the level of a natural chemical modification called methylation, in which a methyl group is attached to specific bits of DNA, genes ordinarily turned off in the course of development stayed active, and the plants developed abnormally. They made more leaves and took longer to start flowering than usual, and then produced abnormal flowers. "We're saying that methylation is required for plant development," says Yale University's Stephen Dellaporta, a co-author of the *Science* report. "What you see is a cause-and-effect relationship, not just an association."

The finding doesn't rule out mechanisms that selectively activate certain genes, but it does support the long-standing suspicion that methylation is one of nature's ways of shackling the activity of genes that become unnecessary as development proceeds. Four years ago, for example, Timothy Bestor, then at Harvard Medical School, found the gene for a methylating enzyme in mice and then, working with Rudolf Jaenisch's team at the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts, crippled it. The resulting "knockout" mice produced embryos that died after only about 9 days. But because the embryos were so short-lived, it was not possible to see how lack of methylation affected their development.

And other species that naturally lack DNA methylation, including yeast and the fruit fly *Drosophila melanogaster*, do just fine without it. So even though researchers have generally found that heavily methylated genes tend to be inactive, doubts remained about whether methylation is in fact critical for gene regulation or the activities, including development, that depend on it. "There was a lot of hand-waving [about methylation]," but no direct evidence of its importance, notes developmental geneticist R. Scott Poethig of the University of Pennsylvania, Philadelphia.

The current work should change that. "It shows that methylation is critically important. It's a milestone in the field," says Aharon Razin, a molecular geneticist at Hebrew University Medical School in Jerusalem, Israel, who has been doing methylation studies for decades. Poethig agrees: "Clearly, this [new information] is a wake-up call for us to think more carefully about what methylation might be doing."



Petal power. Severe reduction in methylation causes the *Arabidopsis* flower to make excess petals and no stamens. The inset photo is normal.

For their experiments, Dellaporta, Michael Ronemus, also of Yale, and their colleagues worked with Jychian Chen from Academia Sinica in Taipei, Taiwan, to first block expression of the gene that codes for cytosine DNA methyltransferase, the enzyme that methylates DNA in *Arabidopsis*. They did this by introducing into *Arabidopsis* plants a reversed copy of the enzyme's gene. That copy produces a so-called antisense RNA that can bind to the normal RNA made by the methyltransferase gene and prevent it from directing synthesis of the enzyme. The researchers expected that synthesis of the enzyme would decrease in proportion to how much antisense was produced, and while they did not measure synthesis itself, the effects observed indicate that this is what happened.

In the modified plants, the researchers saw striking changes in the developmental patterns. Normally, *Arabidopsis* takes about 26 days to reach sexual maturity, passing through several distinct growth stages along the way. In the first or vegetative stage, leaves appear and the stem elongates. After an individual plant has produced eight leaves comes the transition to the inflorescence, in which flower stalks sprout. And finally, there is a shift to the reproductive stage, when flowers appear.

But plants making enough antisense to reduce the methylation of their genomes by 71% took about 47 days to start making flower stalks, during which time each plant produced about 27 extra leaves. They then went on to generate five times the usual number of flower stalks. In addition, the transitions between these developmental stages were sloppier, says Dellaporta. Leaf-making genes were still going strong in some cells even after others had shifted into a flower-production mode. These changes indicated that without the proper amount of methylation, developmental stages were prolonged, so that the actively growing tip of the plant retained its ability to differentiate into a wider variety of tissues for a longer time.

The second team studying methylation and *Arabidopsis* development has come up with results that show "remarkable" parallels to the Yale findings, says one of the researchers, E. Jean Finnegan at the Commonwealth Scientific and Industrial Research Organization (CSIRO) in Canberra, Australia. In work that will appear in the 6 August issue of *Proceedings of the National Academy of Sciences*, Finnegan and her colleagues genetically engineered *Arabidopsis* plants to make the same antisense as in the Yale work. The result was the same pattern of developmental abnormalities, together with some alterations not seen by the Yale group: The flowers of some plants developed extra petals instead of stamens and carpels, and in a few plants, genes for flower development were active in leaves.

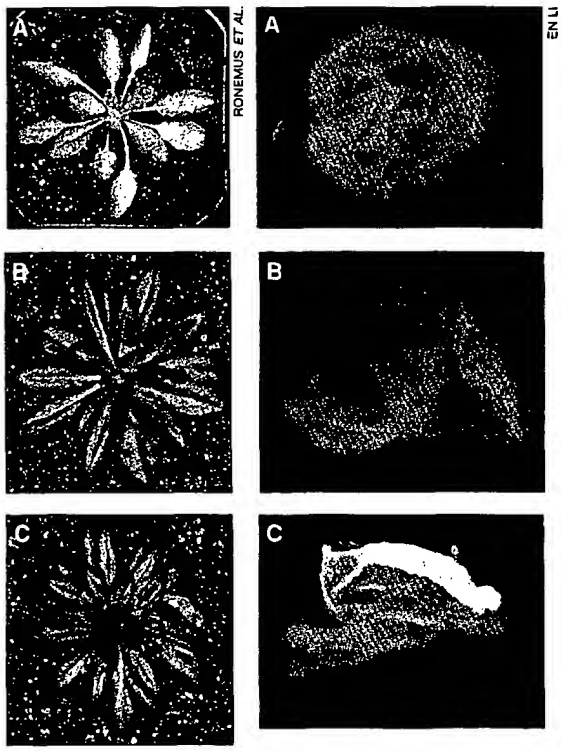
Taking a different tack, molecular biologist Eric Richards, of Washington University in St. Louis, also found that methylation is important for proper *Arabidopsis* development. Richards works with a mutant called *ddm1*, for "*decrease in DNA methylation*." When he first made the mutation, the resulting plants grew almost normally, even though their DNA was poorly methylated. As a result, some researchers had viewed the mutants as evidence against methylation's importance. But that is not the case, Richards says.

In still unpublished work, his team found that when the *ddm1* mutants are interbred, the progeny plants eventually show the same sorts of abnormalities as the antisense plants studied by the Yale and CSIRO groups. The original *ddm1* mutants looked relatively normal, he says, because in that first generation, methylation is lost mainly from repetitive, noncoding DNA, which does not make the proteins needed for forming leaves or flowers. Richards has not yet identified the specific biochemical defect causing low DNA methylation in *ddm1* plants—it apparently does not affect the methyltransferase—but suggests that as the defect is passed from generation to generation, it may lead to ever less methylation, with the result that developmentally important genes are eventually affected.

Indeed, the Yale and CSIRO groups found that the more severe the loss of methylation, the greater the effect on development. Studies of their original antisense plants showed, for example, that those making small amounts of antisense have much more normal growth patterns than those with lots of antisense. Furthermore, crossing two antisense plants yields some progeny with two copies of the antisense gene, one more than the parents. Consequently, their genes undergo even less methylation than those of their parents and, like Richards's *ddm1* mutants, become more abnormal with each passing generation, taking longer to mature and developing extra leaves and flower stalks. In contrast, the progeny of antisense plants and normal parents were still abnormal—as if the antisense parents had passed on their altered methylation patterns—but the progeny's abnormalities were less severe, presumably because the methylating enzyme is back in action.

Mice show a similar correlation between the extent of their methylation problems and the resulting developmental abnormalities. In still unpublished work, Jaenisch's group genetically engineered mice that contain a totally inactivated form of the methylating enzyme, rather than a crippled one. The embryos of these mice, with even more severely lowered gene methylation than the original knockouts, died within 8 days, rather than 9.5 or so, Jaenisch says. While that difference seems small, Bestor notes, "a day and a half [in the life of these embryos] reflects enormous changes."

Just how lack of methylation might disrupt development is less certain, but the Yale and CSIRO teams have come up with a clue. In contrast to Richards's finding that *ddm1* plants primarily lack methyl groups on their noncoding DNA, at least initially, they find that their antisense plants lack methyl groups on coding sequences, including DNA bits called promoters, which help control expression of actual genes, as well as on some noncoding sequences.



Proportional problems. The more drastic the drop in methylation (A to C), the more rosette leaves *Arabidopsis (left)* produces, and the less development mouse embryos *(right)* undergo.

Because experiments with cultured plant cells by other workers had shown that the addition of methyl groups to a gene's promoter can inactivate the gene, Dellaporta and Finnegan propose that in the whole plants, normal methylation of particular promoters turns off genes important to one stage of development, say, leaf formation, opening the way for genes for the next—flower stalk formation—to be expressed until they, too, are turned off by methylation. "[Methylation] is sort of an endogenous clock, a higher order type of regulation," Dellaporta suggests. "It can serve as a signal for the transitions that take place." This idea is consistent with other teams' observations that the DNA of leaves produced early in the plant's life is less methylated than that of later leaves.

Not everyone is ready to embrace that proposal, however. Bestor, now at Columbia University College of Physicians and Surgeons, thinks that methylation is not an endogenous clock at all, but a host defense mechanism. Normally, he argues, methyl groups serve to silence foreign genes that have inserted themselves into the plant genome sometime during its evolutionary history. With the methylating enzyme gone, these genes are able to become active, and their uncontrolled activity could cause the developmental abnormalities seen, he suggests. It will take determining the exact nature of methylated DNA to decide between these hypotheses.

There is debate, too, about whether methylation causes gene inactivation in the first place, or simply reinforces that state. And if methylation does both, then how many enzymes are involved? Jaenisch insists that there are two, one that inactivates the genes and another that keeps adding methyl groups at that spot each time the chromosome is replicated to keep the gene quiet. In support of this, he cites evidence from his knockout mice, in which some methylation goes on even without the one known methyltransferase.

For their part, Dellaporta and Bestor predict that there is one methylating enzyme that plays both roles in gene activation. As evidence, they cite the fact that so far no one has been able to find a second methylating enzyme. What's more, the plant and animal methyl transferases are very similar. So, if the plant enzyme can both maintain methylation patterns and initiate new ones, as it seems to be able to in tests with cultured plant cells, the animal enzyme ought to be able to do the same. "There may be others [methylating enzymes], but they may be very minor," Bestor says.

Israel's Razin thinks that no matter how many enzymes are involved, methylation works with other control mechanisms. He points to evidence from his group and others suggesting that protein factors that bind to DNA are what really shut down the gene, not methylation per se. These factors are known to help regulate gene expression, although exactly how is uncertain. It could be that the protein attaches wherever methylation has occurred and shuts down the gene, or that methylation alters the three-dimensional structure of a chromosome to make the target DNA accessible to these factors. Or the protein may be what enables methylation (and deactivation) to occur at all. Thus, Razin argues, "methylation is not the whole story."

But even if a methylating enzyme is just the genome's assistant librarian, and not the chief, the new results are boosting researchers' appreciation for methylation's role. This simple chemical modification alters how genetic information will be used, without changing the DNA sequence, and that, says Dellaporta, is quite impressive: "It's a beautiful way of changing the gene expression pattern in a stable but reversible fashion."

–Elizabeth Pennisi

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AU Busam, G.; Junghanns, K.T.; Kneusel, R.E.; Kassemeyer, H.H.; Matern, U.
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SO Plant physiology, Nov 1997. Vol. 115, No. 3. p. 1039-1048
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Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 12406–12411, October 1996
Genetics

Developmental abnormalities and epimutations associated with DNA hypomethylation mutations

(epigenetic/DNA methylation mutant/*Arabidopsis thaliana*/*ddm1*)

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Communicated by Ronald L. Phillips, University of Minnesota, St. Paul, MN, August 19, 1996 (received for review May 29, 1996)

ABSTRACT **A number of aberrant morphological phenotypes were noted during propagation of the *Arabidopsis thaliana* DNA hypomethylation mutant, *ddm1*, by repeated self-pollination. Onset of a spectrum of morphological abnormalities, including defects in leaf structure, flowering time, and flower structure, was strictly associated with the *ddm1* mutations. The morphological phenotypes arose at a high frequency in selfed *ddm1* mutant lines and some phenotypes became progressively more severe in advancing generations. The transmission of two common morphological trait syndromes in genetic crosses demonstrated that the phenotypes are caused by heritable lesions that develop in *ddm1* mutant backgrounds. Loss of cytosine methylation in specific genomic sequences during the selfing regime was noted in the *ddm1* mutants. Potential mechanisms for formation of the lesions underlying the morphological abnormalities are discussed.**

DNA modification has been postulated to play a central role in epigenetic regulation by modulating access to the genetic information (1, 2). Much research has centered on the biological significance of the post-replicative addition of a methyl group to the cytosine ring, a DNA modification widespread in both prokaryotes and eukaryotes. The importance of DNA methylation in regulation of a number of cellular processes in prokaryotes, including restriction-modification, transposition, DNA repair, and transcription, is well established (3).

The function of cytosine methylation in eukaryotic cells is less clear. A large body of experimental evidence points to a role for DNA methylation in modulation of gene expression (4, 5). A correlation between increased cytosine methylation and transcriptional quiescence holds for many examples (6), suggesting a role for DNA methylation in maintenance of transcriptional inactivity. Methylation inhibitor (7) and mutant studies (8, 9) have supported the role of DNA methylation in propagation of established transcriptionally quiescent states. However, exceptions exist where increased cytosine methylation is positively correlated with gene activity (8, 10, 11). Moreover, proper gene expression can occur without the contribution of cytosine methylation in some organisms as demonstrated by the existence of eukaryotic species that lack detectable amounts of 5-methylcytosine (12–14).

DNA methylation mutants provide experimental systems to directly address the function of cytosine methylation without the problems associated with correlative studies or the use of methylation inhibitors. The embryo-lethality of the murine engineered DNA methyltransferase mutations clearly demonstrates that cytosine methylation is necessary for completion of early mouse development (15). Further studies with these engineered mutations showed that cytosine methylation is necessary for maintenance of parental imprinting (8) and proper regulation of the X-chromosome inactivation control gene, *Xist* (9). DNA hypomethylation mutants have also been isolated in the filamentous fungus *Neurospora* (16). In contrast to the mouse DNA methyltransferase mutants, *Neurospora* mutants that lack detectable 5-methylcytosine exhibit only mild and variable morphological defects. The different phenotypic consequences of DNA hypomethylation mutations in different organisms point to the diversity with which eukaryotes use DNA modification and the need for parallel studies of DNA methylation in different model systems.

We have been pursuing a genetic approach to an understanding of the function and regulation of eukaryotic DNA methylation through the study of *Arabidopsis thaliana* mutants with altered cytosine methylation. We have previously described the isolation of two independent recessive alleles of the *DDM1* (decrease in DNA methylation) locus that cause an approximately 70% reduction in genomic 5-methylcytosine content (17). The *ddm1* mutations do not map to known cytosine methyltransferase genes (18), nor do they affect cytosine methyltransferase activity detectable in nuclear extracts or metabolism of the methyl group donor, *S*-adenosylmethionine (19). Apparently, the *ddm1* mutations disrupt a novel component of the methylation machinery or affect the methylation of genomic sequences by an indirect mechanism.

Homozygous *ddm1* mutants display only weak morphological changes when first identified in segregating populations (19). Despite the lack of dramatic immediate phenotypes, *A. thaliana ddm1* mutations are associated with the delayed onset of a number of severe developmental abnormalities as described in this report. Our genetic analyses indicate that *ddm1* mutations lead to the formation of heritable lesions at unlinked loci that, in turn, cause the morphological phenotypes.

MATERIALS AND METHODS

Plant Growth. Plants were grown on standard soil mixtures [60% Scotts Redi-earth/40% vermiculite; or 62.5% SupermixA (Sakata, Yokohama, Japan)/25% vermiculite/12.5% perlite) in a greenhouse or in an environmental chamber at 20–25°C under 16–24 hr of illumination per day, with the exception of the clam mapping population, which was grown on axenic solid media (20).

Genetic Mapping. All loci were mapped relative to strain-specific molecular markers (21, 22) in segregating F_2 families. The *BAL* mapping families were generated by a parental inter-strain cross (severe ball *DDM1/ddm1-2* Columbia X wild-type Landsberg erecta strain) that yielded aphenotypic F_1 plants. Four *DDM1/DDM1* F_1 plants, identified by progeny testing, were selfed to generate F_2 mapping populations. The lesion(s) at the *BAL* locus acted in a recessive manner in the inter-strain crosses and the F_2 mapping populations segregated 128 normal: 40 severe ball plants (null hypothesis, $H_o = 3:1$ :: normal: severe ball; $\chi^2 = 0.13$; $P \approx 0.7$). We prepared genomic

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DNA from 26 severe ball F_2 plants and determined genotypes for distributed PCR-based molecular markers to find tight linkage between the lesion at the *BAL* locus and the *AG* marker (22) (2 recombinant chromatids of 52 total). The map assignment was confirmed by demonstration of linkage to another chromosome 4 marker, *PG11* (22) (6 recombinant chromatids of 52 total). Consideration of the *AG* genotypes of the six chromatids showing recombination between *PG11* and *BAL* indicated that the *BAL* locus maps centromere-proximal to *AG*.

The *CLM* mapping families were generated by a parental inter-strain cross (severe clam *ddm1-1/ddm1-1* Columbia X wild-type Landsberg erecta strain) that yielded aphenotypic *DDM1/ddm1-1* F_1 plants. These F_1 individuals were selfed to generate F_2 mapping populations [271 normal: 71 clam plants (H_o = 3:1 :: normal: clam; $\chi^2 = 3.3$; $0.1 > P > 0.05$)]. We prepared genomic DNA from 48 clam F_2 plants and determined genotypes for distributed molecular markers to find linkage to the lesion underlying the clam phenotype. The clam locus (*CLM*) is located in the center of the interval between *GL1* and *BGL1* markers (22) (12 recombinant chromatids of 96 total for each marker).

Using a separate F_2 Columbia/Landsberg mapping population, we mapped the *ddm1-2* mutation to a position approximately 15 centimorgans distal to the *LFY* marker (22) on chromosome 5.

Measurement of Global 5-Methylcytosine Content. The methylation of cytosine in genomic 5′-TCGA-3′ sites was determined by the thin-layer chromatography method of Cedar *et al.* (23), as modified by Vongs *et al.* (17). Total genomic 5-methylcytosine content was determined by digestion of total genomic DNA to nucleotides followed by separation and quantitation by high-performance liquid chromatography (24).

Restriction Digestion and Southern Blot Analysis. *A. thaliana* DNA was prepared using the urea lysis method (25). Restriction digestions were performed using the manufacturers' (New England Biolabs and Boehringer Mannheim) specifications. The digested genomic DNA samples were size-fractionated by agarose gel electrophoresis and transferred to Hybond N+ (Amersham) nylon membranes. The membranes were hybridized with radiolabeled probes prepared by the random priming method (Multiprime system, Amersham) following the high SDS hybridization method of Church and Gilbert (26). The filters were washed at 65°C in 0.2× SSC/0.1% SDS, and the hybridization signals were detected by digital image analysis (FUJIX BAS 2000).

RESULTS

***Arabidopsis ddm1* Mutations Are Associated with the Slow Onset of Severe Developmental Abnormalities.** We noted a high incidence of morphological abnormalities in *ddm1* homozygous lines propagated by repeated self-pollination (Fig. 1 and Table 1). The onset of the abnormalities was strictly associated with the *ddm1* mutations. The *ddm1/ddm1* lines were first backcrossed six times to recurrent parental lines to remove unlinked mutations. Similar morphological defects were conditioned by *ddm1* mutations in at least two genetic backgrounds, Columbia and Landsberg erecta (J.A.J., unpublished). Moreover, the severe developmental defects were seen in selfed lines carrying independently isolated *ddm1* alleles arguing against any contribution from additional mutations closely linked to *ddm1*. To control for the effects of inbreeding, we monitored the phenotypes of 14 *DDM1/DDM1* and *ddm1-2/ddm1-2* lines derived from a single segregating family and propagated by self-pollination in parallel. Plants with abnormal phenotypes were never seen in the *DDM1/DDM1* lines indicating that the onset of abnormal phenotypes was not caused by a non-specific inbreeding depression phenomenon operating in wild-type lines.

A spectrum of morphological abnormalities was seen in the 14 *ddm1/ddm1* mutant lines. Table 1 illustrates the morphological phenotypes scored after six generations of self-pollination. All of the *ddm1/ddm1* lines contained plants exhibiting aberrant morphologies, including reduction or increase in apical dominance, short internode length, late flow-

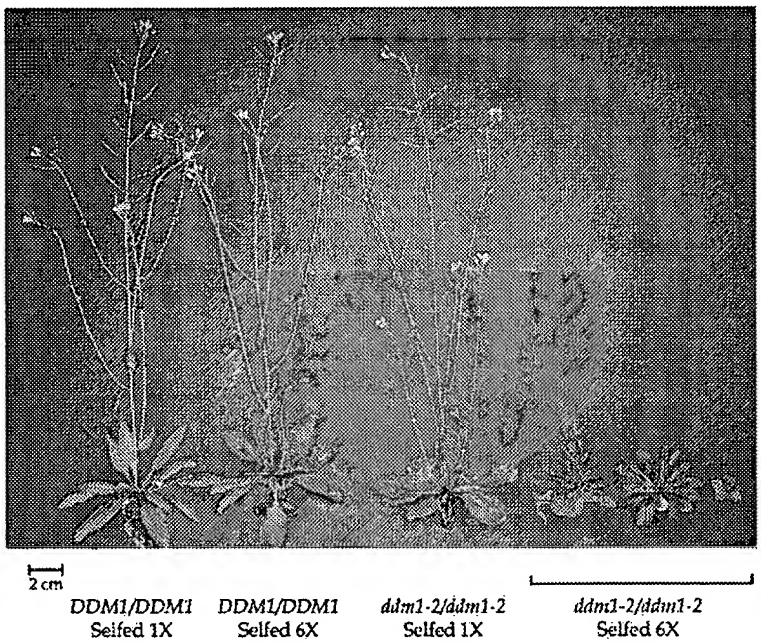

FIG. 1. Morphological phenotypes seen in *ddm1/ddm1* lines propagated by self-pollination. Severe abnormal morphological phenotypes were only seen in *ddm1* mutant lines and only occurred after propagation of the mutant lines by repeated self-pollination. Weak morphological abnormalities, such as slightly reduced apical dominance and rounder leaves (19), were evident in 1× selfed *ddm1* lines. Phenotypes differ among *ddm1* mutant lines (left to right: representatives of lines 1, 2, and 9). All plants were the same age (40 days) and were grown in parallel, under the same environmental conditions.

Table 1. Spectrum of morphological phenotypes and epimutations in 14 *ddm1-2/ddm1-2* and 14 *DDM1/DDM1* control lines after six generations of self-pollination

	Genotype and line number														
	ddm1-2/ddm1-2														*DDM1/DDM1*
Phenotype	1	2	3	4	5	6	7	8*	9	10	11	12	13	14	15–28
Reduced apical dominance†	5/5			3/6	6/6	2/6	1/5	6/6	3/6					1/6	0/84
Short internode	5/5					2/6		6/6	1/6						0/84
Late flowering‡	5/5	5/5	1/6	2/6		2/6		3/6		2/6	5/6		1/6	1/6	0/84
Small leaves§							1/5		2/6		1/6		1/6	1/6	0/84
Increased cauline leaf number¶		3/5		1/6		1/6				4/6	3/6				0/84
Reduced fertility				4/6		1/6	3/5	6/6	3/6	1/6		4/5	1/6	2/6	0/84
Abnormal flowers‖		2/5		1/6							1/6	4/5		2/6	0/84
Epimutation 1	0/4	0/4	4/4	4/4	0/4	4/4	0/4	0/2	0/4	0/4	2/4	0/4	0/4	1/4	0/28
Epimutation 2	4/4	3/4	0/4	0/4	2/4	0/4	0/4	1/2	2/4	2/4	0/4	0/4	2/4	0/4	0/28
Epimutation 3	0/4	4/4	1/4	0/4	4/4	2/4	0/4	1/2	0/4	0/4	4/4	4/4	0/4	4/4	0/28

Fourteen *ddm1-2/ddm1-2* and fourteen *DDM1/DDM1* plants were identified in a segregating population generated by self-pollination of a *DDM1/ddm1-2* heterozygote (6× backcrossed). Independent lines were generated by self-pollination of each of these plants. In each generation, plants were randomly selected for phenotypic examination and seeds from self-pollination of a random individual plant were used as the source of the next generation. After six generations of self-pollination, approximately six individuals were selected at random and scored phenotypically. Plants displaying the morphological phenotypes are indicated by the first number in the fraction, whereas the total number of plants examined is given in the denominator. Blanks indicate that no abnormal phenotype was seen. In addition, we used Southern blot analysis (see Fig. 4) to score plants for the loss of methylation sites recognized by the genomic clones m105 (epimutations 1 and 2) and m118 (epimutation 3). The number of epimutations is indicated over the total number of alleles examined.

*Phenotypes from line 8 were scored after five self-pollinations; the line could not be propagated further because of sterility.

†Several flowering shoots (bolts) are produced simultaneously, in contrast to the dominant primary shoot seen in wild-type *A. thaliana*.

‡Plants flowered more than 15 days later than wild-type. The mean ± standard deviation of the flowering date for the 84 plants in the *DDM1/DDM1* (lines 15–28) = 32.3 ± 1.6 days.

§Rosette leaf length ≤ 20% of wild-type. The mean ± standard deviation of the length of the largest rosette leaf for the 84 plants in the *DDM1/DDM1* (lines 15–28) = 31.2 ± 4.1 mm.

¶More than 10 cauline leaves. The mean ± standard deviation of the cauline leaf number for the 84 plants in the *DDM1/DDM1* (lines 15–28) = 4.2 ± 0.8.

‖Reduced sepal number (lines 2, 4, and 11) or unfused carpels (lines 12 and 14).

ering, small leaf size, increased cauline leaf number, and reduced fertility. In addition, some lines displayed plants with abnormal flowers. Plants with reduced sepal number were noted in 3 of 14 *ddm1/ddm1* selfed lines and plants with hooked and partially unfused carpels were seen in two of the *ddm1/ddm1* selfed lines. After 7 generations of self-pollination, 5 of 14 *ddm1/ddm1* lines exhibited a high degree of sterility or seedling lethality (note that line 8 died out after five generations) (data not shown).

While there were differences in the spectrum of the phenotypes among the 14 *ddm1/ddm1* lines, some of the abnormal characters occurred together. One combination of phenotypes is characterized by an increase in apical dominance, an increase in cauline leaf number, and a delay in time to flowering (lines 2, 10, and 12 in Table 1). Another commonly seen combination of phenotypes, which we refer to as the "ball" syndrome, is characterized by reduced apical dominance, twisted leaves, and small plant size (Fig. 2). The severity of the ball syndrome was progressive with more pronounced phenotypes exhibited by plants in families resulting from higher numbers of self-pollinations (Fig. 2). Variability in the severity of the phenotype among siblings in advanced selfed generations was frequently noted.

Independent Segregation of the Morphological Traits and the Potentiating *ddm1* Mutations. To learn more about the basis of the phenotypes seen in the *ddm1/ddm1* selfed lines, we followed the inheritance of a subset of the phenotypes in genetic crosses. The ball syndrome is inherited as a simple Mendelian monogenic trait. Crosses between *ddm1/ddm1* phenotypic ball plants (strain Columbia) and wild-type Columbia plants yielded plants with normal phenotypes and intermediate ball phenotypes. F_2 generations derived by selfing the phenotypically intermediate plants contained plants with normal, intermediate, and severe ball phenotypes in a 1:2:1 ratio, respectively, suggesting the segregation of a semi-dominant lesion (Table 2). Inheritance of the ball phenotype in the F_2 generation was independent of the segregation of the *ddm1* mutation (Table 2). Starting with a *DDM1/ddm1-2* severe ball F_2 plant, we have generated several severe ball *DDM1/DDM1* lines in which no normal plants were seen through three generations of self-pollination (approximately 50 individuals examined per line per generation; data not shown).

The *ddm1* mutation and the locus responsible for the ball phenotype (*BAL*) were mapped relative to strain-specific genetic polymorphisms in segregating F_2 populations as de-

Table 2. Independent segregation of *ddm1* and a single locus controlling the ball phenotype

Phenotype	Non-mutant *DDM1/_*	Mutant *ddm1-2/ddm1-2*	Totals
Normal	23	7	30
Intermediate ball	45	19	64
Severe ball	18	10	28
Totals	86	36	122

A reciprocal cross between a phenotypic ball *ddm1-2/ddm1-2* plant (strain Columbia) and a wild-type Columbia plant resulted in F_1 *DDM1/ddm1-2* plants, some of which displayed an intermediate ball phenotype. The direction of the cross did not affect the results. Two independent intermediate ball F_1 plants were selfed to generate two segregating F_2 families. Pooled phenotype data from the F_2 families are shown. The ball phenotype was scored as severe (small rosette, twisted leaves, reduced apical dominance), intermediate (medium rosette size with ruffled leaf surface), or absent (normal wild-type). F_2 *ddm1/ddm1* individuals were identified by Southern blot analysis by monitoring the loss of *Hpa*II restriction endonuclease modification of genomic rDNA genes (17). The *ddm1-2* mutation segregates as a single recessive monogenic factor (H_o = 3:1 :: non-mutant: mutant; χ^2 = 1.3; $P \approx 0.25$). The ball phenotype was inherited as a semi-dominant monogenic trait in this intra-strain cross (H_o = 1:2:1 :: normal: intermediate ball: severe ball; χ^2 = 0.36 with df = 2; $P \approx 0.8$). The ball trait and the *ddm1-2* mutation segregated independently (H_o = independent segregation; χ^2 = 1.1 with df = 2; $P \approx 0.5$).

scribed in *Materials and Methods*. The *DDM1* locus maps to the distal portion of the lower arm of chromosome 5, whereas the *BAL* locus maps to the lower arm of chromosome 4 (Fig. 3).

Similar results were obtained when we followed the inheritance of another complex trait, designated "clam," which appeared in *ddm1-1/ddm1-1* selfed lines. This trait is characterized by a small, compressed rosette, reduced internode length, and reduced fertility (data not shown). The inheritance of the clam phenotype in mapping crosses indicated that the trait is caused by a monogenic recessive lesion (see *Materials and Methods*). The locus responsible for the clam phenotype (*CLM*) is also unlinked from the *DDM1* locus and maps to the center of chromosome 3 (Fig. 3).

Progressive Reduction in Cytosine Methylation in Specific Genomic Regions Parallels the Onset of Morphological Phenotypes. The strict association between the DNA hypomethylation mutations and the onset of developmental defects prompted us to look for DNA methylation changes in selfed *ddm1/ddm1* lines. We first measured global DNA methylation levels in *ddm1/ddm1* lines during the selfing regime (see *Materials and Methods*), but the precision of these measurements was too low to reliably detect small changes in methylation levels (data not shown). We subsequently examined specific genomic regions by a more sensitive assay, Southern blot analysis, which revealed a progressive reduction in cytosine methylation during the selfing of *ddm1/ddm1* lines. Although most genomic regions known to be methylated in wild-type *A. thaliana* are hypomethylated in *ddm1* homozygotes, we previously demonstrated that two methylated single-copy regions were unaffected in *ddm1* mutants that had been selfed a limited number of generations (17). These regions are defined by two anonymous genomic clones, m105 and m118, which were identified in a survey of an *A. thaliana* genomic library for clones carrying inserts recognizing methylated *Hpa*II restriction sites (28). Fig. 4 shows that the wild-type *Hpa*II methylation pattern of the m105 locus was not affected in *ddm1/ddm1* lines that had been selfed for only one generation, confirming our previous results (17). However, stochastic loss of cytosine methylation sites at the m105 locus was noted in most *ddm1/ddm1* lines after six generations of self-pollination (Fig. 4). We identified the loss of two separate m105 methylation sites [designated epimutations 1 and 2 in Table 1; epimutations = heritable alterations in DNA modification (2)] in the selfed *ddm1/ddm1* lines (Table 1 and Fig. 4). No m105 epimutations occurred in the selfed *DDM1/DDM1* control lines. Similarly, loss of *Hpa*II site methylation (Table 1, epimutation 3) at the m118 locus occurred in many *ddm1/ddm1* lines, but not in the *DDM1/DDM1* control lines. Although the onset of particular aberrant morphological phenotypes could not be correlated with particular m105 and m118 epimutations, the stochastic loss of DNA methylation sites at these loci paralleled the progressive onset of morphological abnormalities in the selfed *ddm1/ddm1* lines.

DISCUSSION

The results presented here indicate that loss of *A. thaliana* wild-type *DDM1* gene function leads to developmental defects. Previously we demonstrated that *ddm1* mutations, when present in a homozygous state, cause immediate hypomethylation of a large variety of repeated DNA families, as well as low-copy sequences at chromosomal termini (17). In this report, we show that the *ddm1* mutations lead to a slow loss of methylation in non-telomeric single-copy sequences. The DNA methylation system appears to operate differently on the single-copy versus repetitive genomic compartments because methylation of the two sequence classes is differentially affected by *ddm1* mutations.

A variety of morphological anomalies were generated at a high frequency in *ddm1/ddm1* lines propagated through several generations by self-pollination. The onset of the phenotypes was strictly associated with the *ddm1* mutations and never occurred in wild-type sibling lines propagated in parallel with the *ddm1* mutant lines. Although full expression of the phenotype required several generations to develop, once the morphological traits emerged, they were inherited. In each of

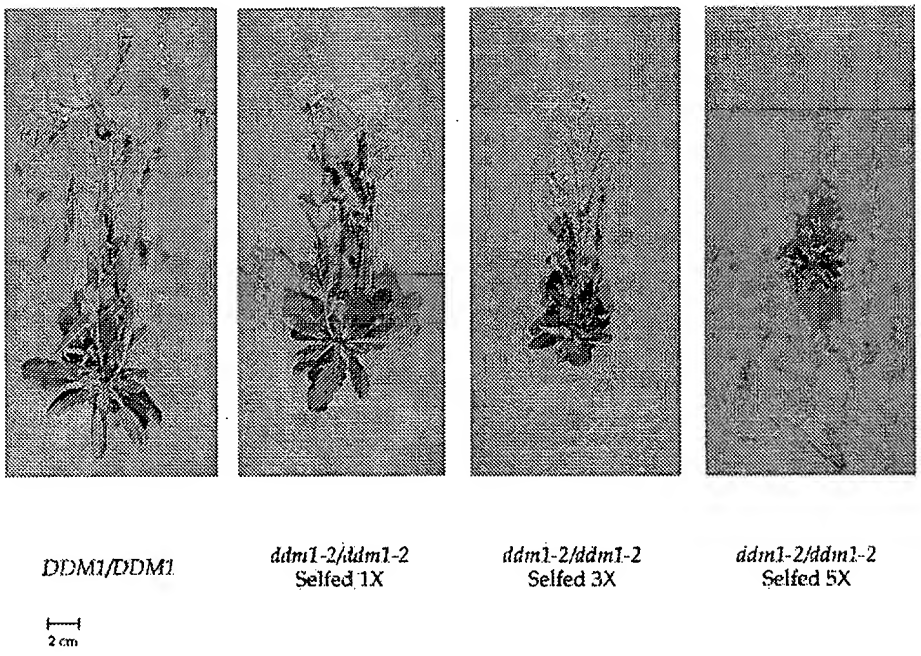

FIG. 2. Progressive onset of the ball phenotype in a *ddm1-2/ddm1-2* line propagated through increasing numbers of self-pollinations. (*Left*) A wild-type *DDM1/DDM1* plant, strain Columbia. All the remaining plants are Columbia *ddm1-2* homozygotes from a single line (not represented in Table 1) propagated by self-pollination. The *ddm1-2* mutant that was selfed once exhibited only mild morphological phenotypes. In the advanced selfed generations, a progressively severe ball phenotype (reduced apical dominance and plant size, twisted leaves) was evident. All plants were the same age (approximately 6 weeks) and were grown in parallel, under the same environmental conditions.

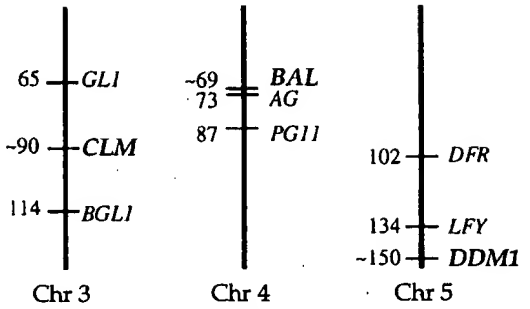

FIG. 3. Map positions of *DDM1* and the loci, *BAL* and *CLM*, affected in *ddm1* mutant backgrounds. The position of relevant reference markers are given (in centimorgans) from the recombinant inbred genetic map of Lister and Dean (27). Approximate positions for the *DDM1*, *BAL*, and *CLM* loci were determined by reference to established markers as described in the *Materials and Methods*.

the two cases examined, the complex morphological trait was caused by a heritable lesion at a single Mendelian locus unlinked to the potentiating *ddm1* mutation.

The heritable lesions underlying the phenotypes could be genetic mutations or epigenetic modifications. Three general mechanisms (not necessarily mutually exclusive) for formation of the lesions are considered here. DNA hypomethylation could lead to an increased mutation rate (model 1) due to increased transposition of previously suppressed elements (30–34) or increased recombination rates (35, 36) mediating genomic rearrangement. However, the characteristics of the phenotypic onset in the selfed *ddm1* homozygous lines cannot be easily explained by random genetic mutation events. The phenotypes occur at a high frequency in *ddm1* homozygous selfed lines, and similar phenotypes occur in independent *ddm1* mutant lines. Moreover, some phenotypes progress in severity as the number of self-pollinations increases.

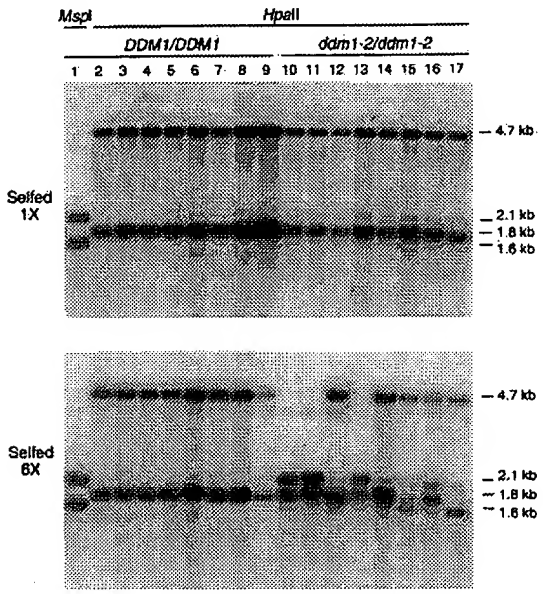

FIG. 4. Progressive loss of cytosine methylation at the m105 locus during propagation of *ddm1/ddm1* plants by self-pollination. Genomic DNA was prepared from leaf tissue from eight different *ddm1-2/ddm1-2* lines (lanes 10–17) and eight *DDM1/DDM1* control lines (lanes 1–9), which were propagated by self-pollination for one generation (*Upper*) or six generations (*Lower*). The DNA samples were digested with the methylation-sensitive restriction endonuclease *Hpa*II (lanes 2–17) or its isoschizomer, *Msp*I, which will cleave C^{m}CGG (29). The digested DNA samples were size-fractionated by agarose gel electrophoresis and transferred to a nylon membrane. The membrane was hybridized with a radiolabeled probe corresponding to a 3.3-kb *Eco*RI subclone from the anonymous single-copy *A. thaliana* genomic clone m105 (28). Epimutation 1 corresponds to the loss of methylation at a *Hpa*II site that converts the 4.7-kb fragment to a 2.1-kb fragment. Epimutation 2 signifies conversion of the 1.8-kb fragment to the 1.6-kb fragment by loss of *Hpa*II site methylation. Faint 2.1- and 1.6-kb bands are visible in the *ddm1-2* mutant lanes after one generation of self-pollination, possibly reflecting some loss of methylation in the vegetative tissue.

The characteristics of phenotypic onset suggest the operation of an epigenetic mechanism. In this context, we consider epigenetic modifications to be mitotically transmissible alterations that affect the expression of the locus without changing primary DNA sequence (2). One possible epigenetic mechanism (model 2) involves alteration in chromatin structure secondary to changes in cytosine methylation (37–39). Position-effect variegation in *Drosophila* provides one example of epigenetic defects based on the propagation of altered chromatin structures (40).

Another possible epigenetic mechanism for the onset of morphological phenotypes in selfed *ddm1* lines is the formation of epimutations (model 3). The slow loss of cytosine methylation in the m105 and m118 loci suggests that similar stochastic methylation site loss could create epimutations in *ddm1* backgrounds at loci distributed throughout the genome. Accumulated loss of multiple methylation sites at a single locus may be responsible for the delayed onset and progressive severity of the morphological defects. The variation in phenotypic severity seen among siblings in selfed populations could be due, in part, to continued creation of new epimutations in somatic tissue followed by transmission to and segregation in the next generation. Our group (17) and others (41, 42) have demonstrated that hypomethylated DNA is inherited in *A. thaliana* across generations due to slow *de novo* methylation. It should be noted that there is precedence for creation and transmission of stable epimutations or epigenetic states in plants (25, 43–48).

It is also possible that the *ddm1* mutations lead to the morphological phenotypes through a DNA methylation-independent pathway, but several considerations suggest that the loss of cytosine methylation is important for the delayed-onset morphological phenotypes. Phenotypes resembling the *ddm1* induced delayed-onset defects are seen in transgenic *A. thaliana* expressing cytosine methyltransferase anti-sense constructs (41, 42). In addition, dwarf-like phenotypes (reduced stature, reduced apical dominance) have been induced in *A. thaliana* (J.A.J., unpublished work) and other flowering plants using DNA methylation inhibitors (49, 50).

We are currently pursuing the molecular characterization of lesions at *ddm1* target loci, such as *BAL*, to determine the mechanism(s) responsible for the morphological defects. We anticipate that these studies will contribute to an understanding of the role of DNA methylation in eukaryotic cells.

We thank our colleagues at Washington University for critical input. We also thank Mike Dyer for greenhouse management. We acknowledge the *Arabidopsis* Biological Resource Center at Ohio State University, and Leonard Medrano and Elliot Meyerowitz for supplying the m105 and m118 clones. J.A.J. is supported by a predoctoral fellowship from the Monsanto Corporation and a training grant from the National Science Foundation (BIR 9256779). This work is supported by National Science Foundation Grant MCB 9306266.

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TI Characterization and expression of caffeoyl-coenzyme A 3-O-methyltransferase proposed for the induced resistance response of Vitis vinifera L.
AU Busam, G.; Junghanns, K.T.; Kneusel, R.E.; Kassemeyer, H.H.; Matern, U.
AV DNAL (450 P692)
SO Plant physiology, Nov 1997. Vol. 115, No. 3. p. 1039-1048
Publisher: Rockville, MD : American Society of Plant Physiologists, 1926-
CODEN: PLPHAY; ISSN: 0032-0889

TI DNA methylation and plant development.
AU Richards, Eric J.
CS Dep. Biology, Campus Box 1137, Washington Univ., One Brookings Drive, St. Louis, MO 63130 USA
SO Trends in Genetics, (1997) Vol. 13, No. 8, pp. 319-323.
ISSN: 0168-9525.

TI Expression of the potato leafroll virus ORF0 induces viral-disease-like symptoms in transgenic potato plants
AU van der Wilk, Frank; Houterman, Petra; Molthoff, Jos; Hans, Fabienne; Dekker, Ben; van den Heuvel, Johannes; Huttinga, Harm; Goldbach, Rob
CS DLO Res. Inst. Plant Protection (IPO-DLO), Wageningen, 6700 GW, Neth.
SO Molecular Plant-Microbe Interactions (1997), 10(2), 153-159
CODEN: MPMIEL; ISSN: 0894-0282

TI Developmental abnormalities and epimutations associated with DNA hypomethylation mutations
AU Kakutani, Tetsuji; Jeddeloh, Jeffrey A.; Flowers, Susan K.; Munakata, Kyoko; Richards, Eric J.
CS Dep. Biol., Washington Univ., St. Louis, MO, 63130, USA
SO Proceedings of the National Academy of Sciences of the United States of America (1996), 93(22), 12406-12411
CODEN: PNASA6; ISSN: 0027-8424

TI Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development.
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CS Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, Australia.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1996 Aug 6) 93 (16) 8449-54.
Journal code: 7505876. ISSN: 0027-8424.

TI Demethylation-induced developmental pleiotropy in Arabidopsis.
CM Comment in: Science. 1996 Aug 2;273(5275):574-5
AU Ronemus M J; Galbiati M; Ticknor C; Chen J; Dellaporta S L
CS Department of Biology, Yale University, New Haven, CT 06520-8104, USA.
NC GM38148 (NIGMS)
SO SCIENCE, (1996 Aug 2) 273 (5275) 654-7.
Journal code: 0404511. ISSN: 0036-8075.

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AU Ronemus M J; Galbiati M; Ticknor C; Chen J; Dellaporta S L
CS Department of Biology, Yale University, New Haven, CT 06520-8104, USA.

Plant Physiol. (1997) 115: 1039–1048

Characterization and Expression of Caffeoyl-Coenzyme A 3-*O*-Methyltransferase Proposed for the Induced Resistance Response of *Vitis vinifera* L.

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Cell-suspension cultures of *Vitis vinifera* L. cv Pinot Noir accumulated resveratrol upon fungal elicitation, and the activity of *S*-adenosyl-L-methionine:*trans*-caffeoyl-coenzyme A 3-*O*-methyltransferase (CCoAOMT), yielding feruloyl-CoA, increased to a transient maximum at 12 to 15 h. CCoAOMT cDNA was cloned from the elicited cells and was shown to encode a polypeptide highly homologous to CCoAOMTs from cells of *Petroselinum* species or *Zinnia* species. The expression of the cDNA in *Escherichia coli* revealed that grapevine CCoAOMT methylates both caffeoyl- and 5-hydroxyferuloyl-coenzyme A and is probably involved in phenolic esterification and lignification. Commercial plant activators induce the disease-resistance response of test plants and are considered to mimic the action of salicylic acid. Among these chemicals, 2,6-dichloroisonicotinic acid and benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester provoke systemic acquired resistance (SAR) and were also shown to induce the expression of class III chitinase in grapevine. The SAR response is classified by an unchanged phenotype of tissues, but the mechanistic basis is unknown. Treatment of the cultured *V. vinifera* cells with either fungal elicitor or low concentrations of salicylic acid and 2,6-dichloroisonicotinic acid, respectively, raised the CCoAOMT or stilbene synthase transcript abundance, suggesting that grapevine is capable of the SAR response, whereas benzo(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester was ineffective. The data imply for the first time (to our knowledge) that the expression of phenylpropanoid genes in grapevine is induced by SAR activators without phenotypic consequences and suggest a role for CCoAOMT and stilbene synthase in the disease-resistance response leading beyond the level of pathogenesis-related proteins as markers of the SAR.

Plants respond to local fungal infection by the activation of various defense measures in the challenged tissues, and the induction of phenylpropanoid pathways appears to play a crucial role in this response (Hahlbrock and Scheel, 1989). The activation causes the short-term accumulation of phenolic metabolites, which might possess potent antimycotic activity as such or may protect the cells indirectly after incorporation into the cell wall. The phenolic materials strengthening the cell wall under these conditions have been collectively addressed in the literature as "lignin-like" compounds, but there is increasing evidence that the reinforcement is based primarily on coumaric and ferulic esters of cell wall polysaccharides or callose (Matern and Kneusel, 1988; Matern, 1991; Iiyama et al., 1994). The reinforcement triggered locally by pathogen invasion (Niemann et al., 1991) or fungal elicitation (Graham and Graham, 1991) causes drastic changes in the rigidity and digestibility of the cell wall and presents a major obstacle to the activities of lytic fungal enzymes (Nicholson and Hammerschmidt, 1992; Matern et al., 1995).

Point inoculations of cucumber plants with *Colletotrichum* ssp. have revealed that fungal challenge, in addition to triggering the local resistance response, is capable of mobilizing the plants' resistance capacities in tissues in advance of the fungus (Kuc, 1982). This "induced immunity" of remote tissues was not associated with phenotypic changes but alerted and predisposed the tissues to a more intensive response upon subsequent infection. The state of enhanced resistance was termed systemic acquired resistance (SAR; Lawton et al., 1993). The communication of tissues in the SAR requires a systemic signaling mechanism, the nature of which has not been elucidated. SA was suggested as an endogenous signal substance in this process (Malamy et al., 1990; Metraux et al., 1990; Rasmussen et al., 1991; Yalpani et al., 1991; Raskin, 1992), and recent reports corroborated the local requirement of SA at the site of the challenge inoculation (Gaffney et al., 1993). However, grafting experiments with transgenic tobacco conceivably ruled out SA as the mobile signal for SAR transmission, and its exact role is still under controversial discussion (Vernooij et al., 1994; Shulaev et al., 1995).

The principal phenomenon of SAR has been confirmed for several plants such as tobacco and Arabidopsis (Ward

[1] The research described in this report was supported by the Deutsche Forschungsgemeinschaft, Fonds der Chemischen Industrie, and Novartis Ltd. (Basel, Switzerland).

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Abbreviations: BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester; CCoAOMT, *S*-adenosyl-L-Met:*trans*-caffeoyl-coenzyme A *O*-methyltransferase (EC 2.1.1.104); COMT, caffeate *O*-methyltransferase (EC 2.1.1.6); INA, 2,6-dichloroisonicotinic acid; OMT, *O*-methyltransferase; PAL, Phe ammonia-lyase (EC 4.3.1.5); SA, salicylic acid; SAR, systemic acquired resistance; STS, stilbene synthase (EC 2.3.1.95); VCH3, *Vitis* acidic class III chitinase (EC 3.2.1.14); WP, wettable powder.

et al., 1991; Uknes et al., 1992) and the transient expression of pathogenesis-related proteins, e.g. glucanase and chitinase, was correlated with the SAR in remote tissues (Binder et al., 1989). Although the experimental proof is still lacking for major crop plants, the application of the SAR concept for crop protection is appealing, and chemicals like INA or BTH, which mimic the action of SA and trigger the SAR response at micromolar concentrations in cucumber, tobacco, or Arabidopsis (Metraux et al., 1991; Kessmann et al., 1994; Friedrich et al., 1996; Lawton et al., 1996) were developed commercially. Plants sprayed with these chemicals revealed no signs of metabolic changes unless inoculated subsequently with a fungus. The incubation of cucumber hypocotyls with INA, for example, caused the enhanced incorporation of cell wall-associated phenolics only upon subsequent inoculation with *Colletotrichum lagenarium* (Siegrist et al., 1994). Similarly, incubation of parsley cell-suspension cultures with INA followed by fungal elicitor treatment initiated an enhanced rate of transcription of PAL and 4-coumarate:CoA ligase (EC 6.2.1.12) mRNAs (Kauss et al., 1992) and these changes were succeeded by phenolic reinforcement of the cell wall (Kauss et al., 1993). Most notably, however, INA alone failed to induce PAL activity (Kauss et al., 1992).

The accumulation of lignin-like materials occurred more rapidly and to a greater extent in leaf tissues in the state of SAR than in control leaves (Hammerschmidt and Kuc, 1982; Hammerschmidt et al., 1985) and the ready incorporation of phenylpropanoid compounds into the polymers under these conditions was demonstrated by Dean and Kuc (1987) on feeding radiolabeled cinnamic acid to cucumber plants challenged with *C. lagenarium*. These results already point to a close link between SAR and the sensitization of phenylpropanoid metabolism, although the fact that the tissue phenotype in the SAR state was unchanged and required an additional fungal inoculation to release the enhanced lignification remains puzzling. The reinforcement with lignin-like materials requires the synthesis of feruloyl- and sinapoyl-CoAs (Walter, 1992; Boudet et al., 1995) and a novel route to these substrates was reported recently based on the sequential methoxylations of 4-coumarate:CoA ligase (Kneusel et al., 1989; Pakusch et al., 1989, 1991; Pakusch and Matern, 1991; Matern et al., 1995). The methyltransferase involved, CCoAOMT, was cloned, and the expression of the enzyme activity appeared to be controlled by multiple parameters (Schmitt et al., 1991; Ye et al., 1994; Grimmig and Matern, 1997). The complex pattern of regulation nourished the idea that CCoAOMT might be a target of modulation in the SAR predisposition of tissues for enhanced lignification, which is compatible also with the fact that the pathway beyond PAL must be particularly considered for SAR regulation (Kauss et al., 1992).

In the course of studies of *Vitis vinifera* L., which is cultivated worldwide with an estimated acreage of 10 million ha (Food and Agriculture Organization of the United Nations, 1987) and devastated frequently by fungal diseases (Agrios, 1997), we became aware of the surprisingly little research dedicated to the resistance mechanisms other than phytoalexin production (Langcake and Pryce, 1976; Melchior and Kindl, 1990; Liswidowati et al., 1991) and the description of lignin-like cell wall polymers (Weber, 1992). Grapevine tissues are recalcitrant to biochemical analysis because of their stiff, leathery texture, as well as their high content of phenolic polymers and organic acids. These difficulties can be overcome, however, by developing appropriate cell cultures. We therefore established a number of cell cultures from hypocotyls of different cultivars, and the cv Pinot Noir turned out to be particularly suitable and was chosen for model studies of the regulation of the phenylpropanoid pathway. The induction of CCoAOMT by fungal elicitation or treatment with SA, INA, or BTH was compared in these cultures with the induction of STS mRNA, which encodes the key enzyme of grapevine phytoalexin biosynthesis (Melchior and Kindl, 1990), or to the expression of VCH3 (Busam et al., 1997), postulated previously as an SAR marker gene in tobacco and cucumber (Ward et al., 1991; Lawton et al., 1994). Based on these studies, we propose that grapevine is also capable of the SAR response and we present a plausible explanation for the mode of predisposition to enhanced phenylpropanoid synthesis and cell wall reinforcement.

MATERIALS AND METHODS

Chemicals, Enzymes, and Materials

Sources of restriction enzymes, vectors, *Escherichia coli* host strains, biochemicals, and chemicals are the same as used by Busam et al. (1997). Caffeoyl-CoA, 5-hydroxyferuloyl-CoA, and 5-hydroxyferulate were synthesized according to the method of Stöckigt and Zenk (1975) and Banerjee et al. (1962). *S*-Adenosyl-L-[*methyl*-^{14}C]Met (1.85–2.29 GBq mmol^{-1}) was purchased from Amersham.

Cell Cultures

Pigmented tissue cultures of *Vitis vinifera* cv Gamay Fréaux were obtained from F. Cormier (FRDC, Agriculture Canada, St. Hyacinthe, Quebec). Unpigmented callus cultures were initiated in dark/light intervals (12/12 h) from *V. vinifera* L. cv Pinot Noir stems plated on SH-agar (Schenk and Hildebrandt, 1972) supplemented with 0.5 mg L^{-1} 2,4-D, 2.0 mg L^{-1} *p*-chlorophenoxyacetic acid, and 0.1 mg L^{-1} kinetin. The calli were transferred to B5 agar (Gamborg et al., 1968) containing 0.1 mg L^{-1} 1-NAA, 0.2 mg L^{-1} kinetin, and 250 mg L^{-1} casein hydrolysate, and suspension cultures were established in the modified B5 medium (40 mL in 200-mL Erlenmeyer flasks) and propagated at 110 rpm on a rotary shaker at 25°C in 12-h dark/light cycles. The rapidly growing cells were routinely subcultured every 12 d. Induction of the cell cultures with commercial yeast extract or plant activators (SA, INA, and BTH) and treatment with WP were described by Busam et al. (1997).

CCoAOMT cDNA Cl ning and Sequencing

A *Vitis* sp. cDNA library of 2 × 10^5 recombinants (Busam et al., 1997) was screened by plaque hybridization at low

stringency using a ^{32}P-labeled CCoAOMT cDNA probe from parsley (Schmitt et al., 1991). Two clones were selected in three rounds of plaque purification, and plasmids (pBluescript SK$^+$) harboring inserts of approximately 1.0 kb were rescued from these clones by the in vivo excision protocol for λZAP (Stratagene). Both strands of double-stranded template DNA were sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using modified T7 DNA polymerase (Sequenase, United States Biochemical) and the universal (M13) and reverse sequencing primers (RP) in addition to sequence-derived primers.

S uthern-Blot Hybridization

Genomic DNA was isolated from the leaves of young *V. vinifera* plants (Steenkamp et al., 1994) and the DNA (10 μg) was digested with *Pst*I, *Eco*RI, *Hin*dIII, or *Bam*HI restriction enzymes. The DNA fragments were separated by electrophoresis on a 0.7% agarose gel and blotted to nylon membranes by downward capillary transfer (Zhou et al., 1994). The blots were hybridized at 65°C overnight with a ^{32}P-labeled *V. vinifera* CCoAOMT cDNA (VCCoAOMT) probe, washed under stringent conditions, and subjected to autoradiography (Sambrook et al., 1989).

Northern-Blot Hybridization

Equivalent amounts of either total or poly(A$^+$) RNA, isolated from the cultured *V. vinifera* cells (Busam et al., 1997) and quantified spectrophotometrically (Gene-quant, Pharmacia), were used for hybridization. The hybridization of northern blots (Busam et al., 1997) was carried out overnight at 42°C with digoxigenin-labeled VCCoAOMT or STS (Melchior and Kindl, 1990) cDNA probes in the presence of 50% formamide.

Heterologous Enzyme Expression

The grapevine CCoAOMT cDNA was 5′ truncated by introduction of an *Nde*I restriction site at the translational start codon and PCR amplification with VCCoAOMT cDNA in pBluescript as template. The PCR product was digested with *Nde*I and *Xho*I and inserted into *pET-21b* (Novagen, Madison, WI) for the expression in *E. coli*. The plasmids containing the correct insert were propagated in *E. coli* BL21(DE3) in the presence of 100 μg mL^{-1} carbenicillin according to the work of Studier et al. (1990). Expression of the *V. vinifera* CCoAOMT was induced by the addition of 1 mM isopropyl-β-thiogalactopyranoside, and the cells were harvested by centrifugation (5000*g*, 10 min) after an additional 3 h of incubation. The bacterial pellet was resuspended in 50 mM sodium phosphate extraction buffer, pH 7.5, containing 1 mM $MgCl_2$, 2 mM DTT, and 10% (v/v) glycerol.

CCoAOMT and COMT Assays

CCoAOMT activity was assayed acc rding to the method of Pakusch et al. (1989) using caffeoyl-CoA or 5-hydroxyferuloyl-CoA as a substrate, and COMT activity was measured under equivalent c nditions with caffeate or 5-hydroxyferulate as substrate. Crude enzyme extracts were prepared by homogenization f deep-frozen *V. vinifera* cells in extraction buffer and clearing by centrifugation (10,000*g*, 10 min).

Purification of *V. vinifera* CCoAOMT Expressed in *E. coli*

Crude enzyme extracts from *E. coli*, prepared by sequential addition of lysozyme and DNase and cleared by centrifugation (15,000*g*, 15 min), were subjected to ammonium sulfate fractionation (30–80%). The pellet was resuspended in extraction buffer, cleared by centrifugation, the supernatant was applied to a Sephracryl S-200 HR column (100 × 5 cm), and CCoAOMT was eluted in extraction buffer. Fractions containing CCoAOMT were concentrated by ultrafiltration and applied to a Mono-Q HR 5–5 column (Pharmacia). The CCoAOMT was eluted with a linear NaCl gradient (0–500 mM). The relative molecular mass and apparent purity of the CCoAOMT was monitored by SDS-PAGE on 14% gels, and proteins in the gel were stained with Coomassie blue. The enzyme purified to homogeneity was used for the determination of substrate specificities.

RESULTS

Growth and Elicitation of *V. vinifera* Cell-Suspension Culture

Preliminary RNA and protein extractions of different grapevine tissues and pigmented cell-suspension culture (F. Cormier, Agriculture Canada, Quebec) resulted in poor yields, since high buffer concentrations (1–1.5 M) were required to neutralize the endogenous acid. Therefore, tissue and cell-suspension cultures were initiated from the leaves and hypocotyls of different grapevines such as *V. vinifera* L. cv Pinot Noir, V. *vinifera* cv Cabernet Franc, or *Vitis rupestris*. An unpigmented cv Pinot Noir culture was eventually chosen for further investigations because the cells showed a fine yellow-white appearance, grew rapidly in suspension, and did not accumulate unusually high amounts of phenols or acids. Accordingly, active enzymes and intact RNA were extracted in satisfactory yields from the cultures using 0.05 to 0.1 M buffers. To our knowledge, cell cultures of this cultivar and the induction characteristics have not been reported previously. The inducibility of the cell-suspension culture was therefore tested with various biotic elicitors, taking the accumulation of the stilbene-phytoalexin resveratrol as a marker of the positive response (Langcake and Pryce, 1976). The cell culture reacted rapidly to the addition of live *Pseudomonas syringae* pv *syringae* cells or commercial yeast extract. Resveratrol and related phenolics started to accumulate after about ′ to 9 h under these conditions, and the yellow-white cells and the culture br th turned brown (data not shown). In contrast to many other plant cells (Tietjen et al., 1983; Graham and Graham, 1991), however, the *Vitis* sp. cells apparently failed to respond to the addition of *Phytophthora megasperma* f. sp. *glycinea* cell wall elicitor. Nevertheless, the results documented that *V. vinifera* L. cv Pinot Noir cell

culture can be used for model studies of the molecular mechanism of the grapevine defense response.

Induction of CCoAOMT and COMT Activities

COMT and, in particular, CCoAOMT activities have been commonly considered as indicators of phenylpropanoid synthesis and lignification (Ye et al., 1994; Boudet et al., 1995; Matern et al., 1995). Low constitutive activities of both CCoAOMT and COMT were determined for the cultured grapevine cells (Fig. 1). Following the addition of yeast extract, however, the CCoAOMT activity increased with a lag of about 6 h to reach approximately 3-fold levels within 24 h (Fig. 1A). COMT activity was not induced to a significant extent under these conditions but increased steadily over the time of the experiments by a factor of 1.5 to 2.0 (Fig. 1B). COMT and CCoAOMT from other plants, reportedly methylate caffeic and 5-hydroxyferulic substrates (Matern et al., 1995; Meng and Campbell, 1996), and the corresponding activities were therefore measured in the crude grapevine extracts. Caffeate and 5-hydroxyferulate were methylated by COMT(s) at a constant ratio of approximately 1:3 (Fig. 1B), whereas the CCoAOMT activities with caffeoyl-CoA and 5-hydroxyferuloyl-CoA were coincidentally induced, although the relative ratio varied over the time of the experiments from 0.7 to 0.5 (Fig. 1A). The differential ratio appears to be strongly influenced by the presence of unidentified factors in the crude extracts, since rebuffering of the enzyme by passage through a Sephadex G25 column inverted the ratio of specific activities while the basic time course of induction remained the same (data not shown).

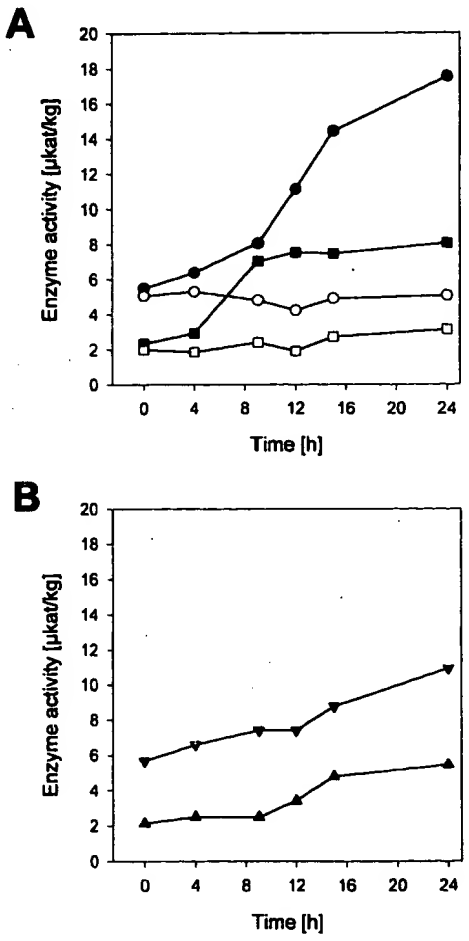

Figure 1. Induction of CCoAOMT (A) and COMT (B) activities in *V. vinifera* cell-suspension cultures. CCoAOMT assays were carried out with *S*-adenosyl-L-(*methyl*-^{14}C)-Met and caffeoyl-CoA (■, □) or 5-hydroxyferuloyl-CoA (, ○), whereas caffeate (▲) and 5-hydroxyferulate (▼) were used as co-substrates in the COMT assays. The cell cultures were treated with yeast extract (1 mg mL^{-1} culture) (■, , ▲, ▼) and untreated cultures served as a control (□, ○).

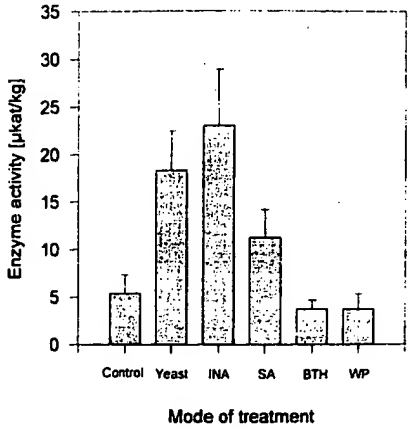

Figure 2. Specific CCoAOMT activities of noninduced (control) versus induced *V. vinifera* cells. The cell-suspension cultures were treated for 24 h with commercial yeast extract (Yeast, 1 mg mL^{-1} culture), 25 μM INA in WP, 20 μM SA, or 25 μM BTH in WP, and the enzyme activities were determined with caffeoyl-CoA. WP (0.15 mg/40 mL culture) lacking an active ingredient was used as a further control.

The induction of CCoAOMT activity in grapevine cells upon treatment with yeast extract served as a control for the induction of the cell cultures with chemicals (INA and BTH) that have been synthesized commercially as plant activators of the SAR response (compare Busam et al., 1997). The specific CCoAOMT activity was determined after 24 h of treatment or in nontreated cultures (Fig. 2), and treatment of the cells with low concentrations of SA served as a means of further control. In contrast to the elicitation with yeast extract, treatment with either INA or BTH did not cause the discoloration of the grapevine cultures. Formulated INA and BTH had to be used in the experiments and the formulation additive itself (WP) was used as an additional control (Fig. 2). Yeast extract or INA turned out to be the most potent elicitors of CCoAOMT activity, with average induction factors of 3 and 4, respectively, whereas SA caused a weaker elicitation. Treatment of the cell suspensions with BTH did not induce CCoAOMT activity. The data clearly indicated that grapevine cells can be induced, albeit in a differential mode, by typical inducers of the SAR response and suggested that the induction of phenylpropanoid pathways beyond the formation of cin-

namic acid (Kauss et al., 1992) might be involved in the response.

CCoAOMT cDNA and Gene Copy Number

Based on the induction timing of CCoAOMT activity (Fig. 1), a cDNA library was generated from poly(A$^+$) RNA of grapevine cells that had been elicited for 4 h with yeast extract, and two clones were selected in three rounds of screening with a parsley CCoAOMT cDNA probe (Schmitt et al., 1991). These clones harbored an identical cDNA insert of 976 bp, designated VCCoAOMT. The insert appeared to represent the full-size cDNA encoding one polypeptide of 242 amino acids (Fig. 3) and containing 5′ and 3′ flanking regions of 59 and 191 bp, respectively, with several putative polyadenylation sites followed by a short stretch of poly(A) (Fig. 3). The size of the insert was compatible with the transcript length of *V. vinifera* mRNA of approximately 1.15 kb determined by northern hybridization, assuming an average poly(A) tailing. Furthermore, a mass of 27,233 D was calculated for the translated polypeptide, which compares favorably to CCoAOMTs from other plants, and database alignments with CCoAOMTs from

```
1                                                          GGCA
5      CGAGGAGAAAGTTTCGGGCGAATACTAGAAGGAAAGGAACAGAAGATCTCTAGAA

60     ATG GCC ACG AAC CAA GAA GCT GGG AGG CAC CAG GAG GTT GGC
       M  A  T  N  Q  E  A  G  R  H  Q  E  V  G                14

102    CAC AAG AGC CTT TTG CAG AGT GAT GCT CTT TAT CAG TAT ATA
       H  K  S  L  L  Q  S  D  A  L  Y  Q  Y  I                28

144    CTT GAA ACC AGT GTG TAC CCA AGA GAG CCT GAA TCC ATG AAG
       L  E  T  S  V  Y  P  R  E  P  E  S  M  K                42

186    GAG CTC AGA GAG TTG ACT GCC CAG CAT CCA TGG AAC ATC ATG
       E  L  R  E  L  T  A  Q  H  P  W  N  I  M                56

228    ACT ACG TCT GCT GAT GAA GGG CAG TTC TTG AAC ATG CTT CTC
       T  T  S  A  D  E  G  Q  F  L  N  M  L  L                70

270    AAG CTC ATC AAT GCC AAG AAC ACC ATG GAG ATA GGC GTC TAC
       K  L  I  N  A  K  N  T  M  E  I  G  V  Y                84

312    ACT GGC TAC TCT CTT CTG GCC ACA GCC CTT GCT CTC CCC GAT
       T  G  Y  S  L  L  A  T  A  L  A  L  P  D                98

354    GAC GGA AAG ATC CTG GCT ATG GAC ATC AAC AAA GAA AAT TAC
       D  G  K  I  L  A  M  D  I  N  K  E  N  Y               112

396    GAG CTG GGT CTG CCA GTA ATT CAA AAG GCA GGG GTT GCC CAC
       E  L  G  L  P  V  I  Q  K  A  G  V  A  H               126

438    AAG ATT GAC TTC AAA GAA GGC CCT GCT TTG CCT GTT CTT GAT
       K  I  D  F  K  E  G  P  A  L  P  V  L  D               140

480    CAG ATG ATC GAA GAT GGC AAG TAT CAC GGG TCG TTC GAC TTC
       Q  M  I  E  D  G  K  Y  H  G  S  F  D  F               154

522    ATA TTC GTG GAC GCA GAC AAG GAC AAT TAT CTG AAC TAC CAC
       I  F  V  D  A  D  K  D  N  Y  L  N  Y  H               168

564    AAG AGA TTG ATC GAT TTG GTG AAG GTG GGG GGA ATC ATC GGC
       K  R  L  I  D  L  V  K  V  G  G  I  I  G               182

606    TAC GAC AAC ACC CTC TGG AAC GGG TCG GTG GTG GCT CCG CCC
       Y  D  N  T  L  W  N  G  S  V  V  A  P  P               196

648    GAT GCT CCG CTG CGG AAG TAC GTG AGG TAC TAC AGA GAC TTC
       D  A  P  L  R  K  Y  V  R  Y  Y  R  D  F               210

690    GTG TTG GAG CTG AAC AAG GCT CTT GCT GCT GAC CCA AGA ATC
       V  L  E  L  N  K  A  L  A  A  D  P  R  I               224

732    GAG ATC TGT ATG CTT CCG GTT GGT GAC GGG ATC ACC CTT TGC
       E  I  C  M  L  P  V  G  D  G  I  T  L  C               238

774    CGT CGG CTA AGC TGA ATCTCCTCGTCAACTCCACCGACGGTGGCTCTTTG
       R  R  L  S  *                                          242

824    ATCGGCGACTCAAAAACTCAATGGAAAATTGATACGACTATTTGCTTTCTTTAAT
879    TTTCCTCTTTTGCGACTTTAACTTTGGATTTGTATTTCAACGAAATGGATGATTT
934    TTTATAATAATAATAATAATAATTAAAAAAAAAAAAAAAAAA
```

Figure 3. cDNA and translated polypeptide sequences of *V. vinifera* CCoAOMT. The nucleotide and amino acid residues are numbered in the left and right margins, respectively. The first polyadenylation site is underlined, and the termination codon is marked by an asterisk.

```
VvCCoAOMT  MATN-----Q EAGPHQEVGH KSLLQSDALY QYILETSVYP REPESMKELR   45
PcCCoAOMT  **S*GESKHC ----------**** ********** ********** ****A*****   44
ZeCCoAOMT  ***PTGET-* P*-K****** ********** ********** ***QP*****   48
MsCCoAOMT  ****ELQKQT *S******** ********** ********F* **H*A*****   50
PtCCoAOMT  ****GEEQQS Q********* ********** ********** ****C*****   50

VvCCoAOMT  ELTAQHFHNI MTTSADEGQF LNMLLKLINA KNTMEIGVYT GYSLLATALA   95
PcCCoAOMT  *V**K****L ********** ********** ********** **********   94
ZeCCoAOMT  FI**K****L ********** **L******* ********** *****S****  100
MsCCoAOMT  *V**K***** ********** *S******** ********** **********  100
PtCCoAOMT  *V**K***** ********** *******V** ********** **********  100

VvCCoAOMT  LPDDGKILAM DINKENYELG LPVIQKAGVA HKIDFKEGPA LPVLDQMIED  145
PcCCoAOMT  ********** ***R****I* **I*E****G *****R**** *****H*L**  144
ZeCCoAOMT  **E******L ***R****I* **I******* *****R**** **L****LQ*  148
MsCCoAOMT  I*E******* ********** ****K****D *****R**** *****E**K*  150
PtCCoAOMT  I*E******* ***P****** ********** ********** **********  150

VvCCoAOMT  GKYHGSFDFI FVDADKDNYL NYHKRLIDLV KVGGIIGYDN TLWNGSVVAP  195
PcCCoAOMT  *****T***V ********** ********** *I**L***** *******AQ*  194
ZeCCoAOMT  E*C******* ********** ********** *F**V***** ******L***  198
MsCCoAOMT  E*N***Y*** ********** ********** ****V***** **********  200
PtCCoAOMT  ********** **********I ********E** ****L***** **********  200

VvCCoAOMT  PDAPLRKYVR YYRDFVLELN KALAADPRIE ICMLPVGDGI TLCRRLS.     242
PcCCoAOMT  A***M***** ******I*** ********** *********V *****I*.     241
ZeCCoAOMT  A********** ********** ****V***V* **Q******* *****I*.     245
MsCCoAOMT  ********** ********** ****V***** ********** *I***IK.     247
PtCCoAOMT  ****M***** ********** ********** ********** ****IQ.     247
```

Figure 4. Alignment of CCoAOMT polypeptide sequences of *V. vinifera* L. (VvCCOAMT, GenBank accession no. Z54233), *P. crispum* (PcCCOAMT, GenBank accession no. M69184), *Z. elegans* (ZeCCOAMT, GenBank accession no. U13151), *M. sativa* (MsCCOAMT, GenBank accession no. U20736), and *P. tremuloides* (PtCCOAMT, GenBank accession no. U27116). Amino acid residues are numbered in the right margin. Asterisks indicate identical amino acid residues, and hyphens bridge the gaps introduced to maximize the alignment.

Petroselinum crispum (Schmitt et al., 1991), *Zinnia elegans* (Ye et al., 1994), *Medicago sativa* (Sewalt et al., 1995), or *Populus tremuloides* (Meng and Campbell, 1995; Fig. 4) revealed more than 70% identity at the nucleotide and 85 to 94% identity at the polypeptide sequence levels. Nonconservative replacements of amino acids were particularly distinct in the N-terminal decapeptide (Fig. 4). Much less identity, about 55% at the polypeptide level, was observed with CCoAOMTs from *Stellaria* spp. (Zhang et al., 1995) or Arabidopsis (Zou and Taylor, 1994) and no significant homology was observed to COMTs or any other plant OMT. The cDNA isolated from the elicited grapevine cells was thus likely to encode the CCoAOMT.

The copy number of grapevine CCoAOMT genes was estimated by Southern hybridization of DNA isolated from leaves of young *V. vinifera* plants, and the patterns of *Pst*I, *Eco*RI, *Hin*dIII, or *Bam*HI restrictions were analyzed using VCCoAOMT cDNA as the hybridization probe (Fig. 5). VCC AOMT cDNA lacks restriction sites for these endonucleases. The small number of restriction fragments, showing three bands in the case of *Hin*dIII and only two bands for *Pst*I, *Eco*RI, and *Bam*HI (Fig. 5), suggested that *V. vinifera* encodes only one or two CCoAOMT genes that may represent the alleles of the same gene. This assump-

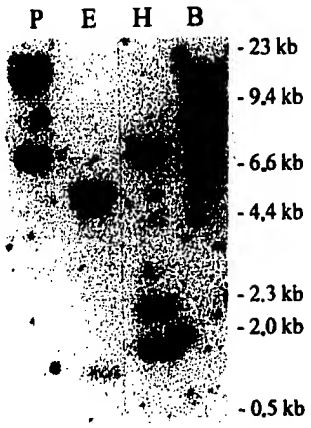

Figure 5. Southern hybridization of genomic DNA isolated from the leaves of young *V. vinifera* plants. The DNA (10 μg/lane) was restricted with *Pst*I (P), *Eco*RI (E), *Hin*dIII (H), or *Bam*HI (B) prior to separation on a 0.7% agarose gel, and ^{32}P-labeled VCCoAOMT cDNA was used as a hybridization probe.

tion is relevant for the bifunctionality of the methyltransferase and the relative ratio of catalysis with the substrates caffeoyl- and 5-hydroxyferuloyl-CoA.

Heterologous Expression of *V. vinifera* CCoAOMT

The VCCoAOMT cDNA was 5′ truncated for the expression in *E. coli* as had been accomplished with enzymes from *Ruta* spp. and *Petroselinum* spp. (Junghanns et al., 1995; Matern et al., 1995; B. Grimmig, unpublished data) and an *Nde*I-restriction site was generated at the start of translation. The amplified cDNA construct was introduced into vector *pET-21b* and transformed into the host strain *E. coli* BL21 (DE3) for isopropyl-β-thiogalactopyranoside-induced expression of CCoAOMT. The heterologous expression yielded a highly active CCoAOMT that was purified on a preparative scale by conventional column chromatography. Subsequent examination by SDS-PAGE revealed one band of 27 ± 2 kD for the homogeneous enzyme (Fig. 6). The catalytic activity of the heterologously expressed grapevine CCoAOMT confirmed the identity of the VCCoAOMT cDNA clone and this pure enzyme was used for the re-evaluation of substrate specificity.

Recent investigations in *Zinnia* spp. (Ye et al., 1994) revealed that CCoAOMT is also involved in the lignification of tissues during ontogenetic development. Woody plant species like *Zinnia* or *Vitis* produce syringyl/guaiacyl-type lignins, which require feruloyl-CoA as well as sinapoyl-CoA substrates for the synthesis of monolignols (Lewis and Yamamoto, 1990; Walter, 1992) and hence the methylation of caffeoyl- and 5-hydroxyferuloyl-CoA. The grapevine CCoAOMT expressed in *E. coli* efficiently methylated caffeoyl-CoA and 5-hydroxyferuloyl-CoA at a relative ratio of about 3:1 (36.5 versus 12.4 mkat/kg) (Table I), which is in contrast to the activities measured with crude *Vitis* sp. cell extracts. Most notably, neither caffeate nor 5-hydroxyferulate was accepted as a substrate, although the specific activity of the enzyme exceeded that of the enzyme isolated from grapevine cells by about 3 orders of magnitude (Table I). The bifunctionality toward caffeoyl- and 5-hydroxyferuloyl-CoA in vitro obviously als qualifies grapevine CCoAOMT fo: the lignification of tissues. Nevertheless, COMT may be additionally involved in vivo in the formation of syringyl units as has been shown in tobacco (Atanassova et al., 1995).

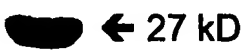

Figure 6. SDS-PAGE separation of purified *V. vinifera* CCoAOMT expressed in *E. coli* (approximately 4 μg). The gel (14%) was stained with Coomassie blue, and the arrow marks the mobility of a 27-kD reference protein.

Expression of CCoAOMT and STS Genes in *V. vinifera* Cells Responding to SAR Activators

The cDNAs of CCoAOMT and STS (Melchior and Kindl, 1990) were used as specific probes to monitor changes in the amounts of transcripts. Treatment of the grapevine cell cultures with yeast elicitor increased the CCoAOMT and STS mRNA levels with equivalent kinetics to a transient maximum at 4 h (Fig. 7). The results are compatible with the time course of induction of the corresponding enzyme activities as well as with the accumulation of the stilbenphytoalexin resveratrol. The induction of grapevine cells with yeast extract again served as a control to compare the effect of plant activator chemicals on de novo transcripti n.

In parallel sets of experiments, the grapevine cell cultures were treated with low concentrations of INA or BTH, which reportedly induce the SAR response in plants (Metraux et al., 1991; Friedrich et al., 1996), as well as with SA, which is under examination as an endogenous signal sub-

Table I. *Specificity of V. vinifera CCoAOMT expressed in E. coli*[a]

Substrate	Enzyme Activity
	mkat kg^{-1}
Caffeoyl-CoA	36.5
5-Hydroxyferuloyl-CoA	12.4
Caffeate	0
5-Hydroxyferulate	0

[a] The assays were carried out with the enzyme purified to homogeneity and representing about 20% of the total *E. coli* protein.

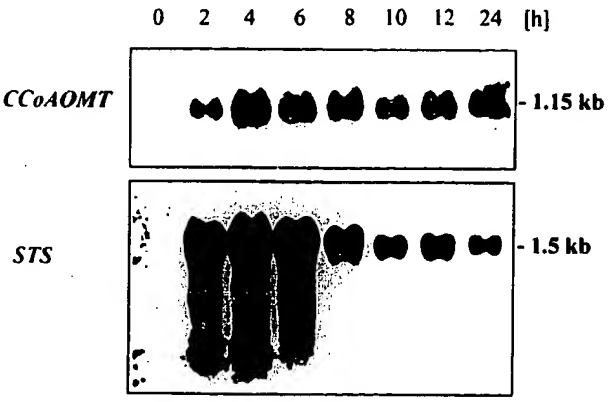

Figure 7. Relative abundance of CCoAOMT and STS transcripts in *V. vinifera* cell cultures induced with crude yeast extract (1 mg mL^{-1} culture). The cells were harvested at various times following the addition of yeast elicitor, and the total RNA was extracted and subjected to northern-blot analysis (7.5 μg/lane) using digoxigenin-labeled cDNAs of VCCoAOMT or STS as hybridization probes.

stance of the local resistance expression (Shulaev et al., 1995). Neither of these treatments caused visible signs of stress in the cultures, which is in contrast to the yeast or *Pseudomonas* sp. elicitor treatments. BTH failed to induce the de novo transcription of STS or CCoAOMT (Fig. 7), whereas treatment of the cell cultures with SA caused a transient, marked increase within 4 h in the amounts of STS and, to a lesser extent, CCoAOMT transcripts (Fig. 8). However, induction of the grapevine cells with INA resulted in the considerable, rapid increase of both CCoAOMT and STS transcript abundances, which reached maximal values after approximately 2 h. During the following 20 h, the CCoAOMT and STS mRNA levels decreased again at a slow rate (Fig. 8). Formulated WP of BTH and INA had to be used in these experiments and the control with formulation material lacking the active ingredient showed no inductive effect on CCoAOMT or STS transcription (data not shown). In tomato and cucumber, the systemically enhanced expression of pathogenesis-related proteins, in particular chitinase and glucanase, had been reported (Binder et al., 1989) and a class III chitinase was proposed as an SAR marker gene in tobacco and cucumber (Ward et al., 1991; Lawton et al., 1994). The plant activators INA and BTH also caused the induction of grapevine VCH3 transcript abundance (Busam et al., 1997). Overall, these results confirmed that grapevine cells respond to SA, INA, or BTH treatment like tobacco or cucumber and suggest that *V. vinifera* may be capable of expressing the SAR response.

DISCUSSION

C mparison of *V. vinifera* CCoAOMT to Heterologous CCoAOMTs

V. vinifera conceivably encodes only one CCoAOMT from one or two genes (Fig. 5) and the corresp nding cDNA (VCCoAOMT) was cloned (Fig. 3) and identified by sequence alignments (Figs. 3 and 4) with CCoAOMTs from various other plants (Ye et al., 1994; Schmitt et al., 1991; Meng and Campbell, 1995; Sewalt et al., 1995) as well as by the catalytic activity of the heterologously expressed enzyme (Fig. 6; Table I). The sequence of *V. vinifera* CCoAOMT clearly differed from those of COMTs (Meng and Campbell, 1996) and other plant OMTs. The homologous sequence and the low gene copy number of *V. vinifera* CCoAOMT are reminiscent of the parsley CCoAOMT (Schmitt et al., 1991; Grimmig and Matern, 1997), but differ from *Zinnia* spp. (Ye et al., 1994), which encodes a family of genes, or *Stellaria* spp. and Arabidopsis (Zou and Taylor, 1994; Zhang et al., 1995), which share much lower homologies. The highly active VCCoAOMT, expressed in *E. coli* by a protocol that had been used for the preparative expression of CCoAOMTs from parsley or acridone synthase from the common rue (Junghanns et al., 1995; Matern et al., 1995), catalyzed the methylation of both 5-hydroxy-feruloyl- and caffeoyl-CoA but was inactive with caffeate or 5-hydroxy-ferulate, just like the parsley CCoAOMT (B. Grimmig, unpublished data). COMTs, for comparison, appear to be less specific and occasionally methylate also caffeoyl-CoA, as observed in poplar (Meng and Campbell, 1996) but not in alfalfa (Sewalt et al., 1995).

Crude extracts from grapevine cells apparently contained unidentified components that shift the relative ratio

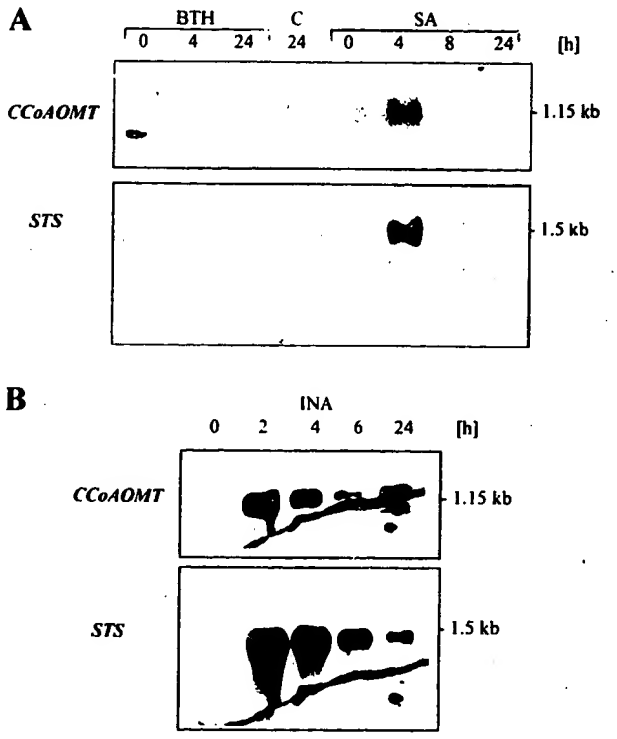

Figure 8. Relative amounts of CCoAOMT and STS transcripts in *V. vinifera* cell cultures treated with 25 μM BTH (A) or 20 μM SA and 25 μM INA (B). The cells were harvested at various times of treatment and the total RNA (A) or the poly(A$^+$) RNA (B) was extracted and subjected to northern-blot analysis (7.5 μg total or 0.7 μg poly[A]$^+$ RNA per lane) using digoxigenin-labeled cDNAs of VCCoAOMT or STS as hybridization probes. Control cultures were treated for 24 h with WP lacking BTH (lane C 24 h).

of substrate specificities in favor of 5-hydroxyferuloyl-CoA (Fig. 1), whereas the enzyme expressed in *E. coli* (Table I) or the crude enzyme after Sephadex gel filtration preferred caffeoyl-CoA over 5-hydroxyferuloyl-CoA by a factor of 3, and this effect deserves further attention. The gel-filtration experiments, in particular, ruled out that low-homology CCoAOMTs, which might have escaped the hybridization, were responsible for the observed shift in specificity, since low-molecular-weight factors rather than different enzyme entities determined the shift in specificity. The experiments indirectly also refuted the possibility of hydrolysis of the CoA-ester substrates during the assay prior to methylation of the acids by COMT activities (different inducibilities of COMT and CCoAOMT activities), although the definitive proof would require more sophisticated enzyme assays.

CCoAOMT Is Involved in the Induced Resistance Response of *V. vinifera*

Cell cultures of *V. vinifera* qualified for the model investigations by their selective inducibility with yeast extract and the convenient recovery of enzymes and RNA. Treatment of the cells with yeast elicitor coordinately induced the transcript abundance and activity of CCoAOMT (Figs. 1 and 7), and the mRNA induction pattern was very similar to that of the STS, which was monitored from the same filter blots. STS catalyzes the pivotal reaction in the synthesis of the *V. vinifera* phytoalexin resveratrol and has been extensively studied for its role in disease resistance and stress compensation (Hain et al., 1993). STS and CCoAOMT activities rely on the same substrates and the coordination of their induction documents the synergistic requirements for full expression of the disease resistance in grapevine. *V. vinifera* thus differs from alfalfa, in which de novo transcription of CCoAOMT seems to occur without consequent translation (Ni et al., 1996). The accumulation of lignin-like materials for cell wall reinforcement was correlated with resistance in several host-pathogen interactions (Hammerschmidt et al., 1985; Tiburzy and Reisener, 1990; Reimers and Leach, 1991), which appears to apply also to grapevine (Weber, 1992), and the physiological significance of CCoAOMT for cell wall reinforcement has been outlined in other plant systems (Schmitt et al., 1991; Matern et al., 1995). Thus, the induction of CCoAOMT is an integral part of the disease-resistance response of *V. vinifera* and follows the kinetics of other enzymes of the inducible phenylpropanoid pathway.

Chemical Induction of the SAR

The development of synthetic plant activators that induce the SAR response has added a new perspective to the protection of commercial crops. The effects of INA or SA as chemical inducers have been studied experimentally (Metraux et al., 1990, 1991), whereas the more selective BTH was released only recently (Friedrich et al., 1996; Lawton et al., 1996) for the application in wheat, tobacco, banana, and tomato. The efficiency and m de of action of these chemicals in special crops such as grape, however, has not yet been investigated. In a preliminary study, grape plantlets were sprayed with INA 10 and 4 d prior to the inoculation with *P. viticola*, the causal agent of downy mildew disease, and these studies revealed a significantly enhanced resistance of the INA-pretreated plants as compared with water controls (G. Busam, unpublished). The slow growth of grapevine plants and the harsh conditions required for tissue extraction, however, are hurdles for biochemical investigations, and the experiments were therefore continued with the newly initiated *V. vinifera* L. cv Pinot Noir cell cultures. The expression of pathogenesis-related proteins, in particular chitinases (Legrand et al., 1987), had been recommended as a molecular marker of the SAR response in plants (Binder et al., 1989; Ward et al., 1991). Accordingly, VCH3 (Busam et al., 1997) was used to probe the induction. Treatment of the *Vitis* sp. cell cultures with low concentrations of INA, BTH, or SA induced rapid and long-lasting increases in the VCH3 transcript level (Figs. 8 and 9), whereas the general appearance of the cell cultures remained unchanged. This might be considered a first, preliminary indication that grapevine is capable of the SAR response.

Treatment of the cultures with INA but not with BTH triggered concomitantly the de novo expression of CCoAOMT and STS mRNAs (Figs. 8 and 9), whereas SA caused only a low-intensity, transient signal at 4 h, which may be due in part to rapid turnover, i.e. by glucosidation, as known from tobacco (Lee et al.. 1995). The notion of CCoAOMT and STS induction by INA (Fig. 9) is particularly remarkable with respect to the unchanged phenotype of th cells. Furthermore, INA and BTH display differential modes of action, which overlap in the induction of VCH3 (Figs. 8 and 9) but differ greatly in the induction of phenylpropanoid enzymes such as CCoAOMT or STS (Figs. 8 and 9), which are indispensable for the expression of resistance (Dean and Kuc, 1987; Hammerschmidt and Kuc, 1982; Nicholson and Hammerschmidt, 1992; Hain et al., 1993). This questions the reliability of the induction of pathogenesis-related proteins as an indicator of the SAR of plants.

Putative Role of CCoAOMT in the SAR Response of *V. vinifera*

Previous studies by Kauss et al. (1992) had revealed that PAL was not induced by INA treatment of parsley cells. The combination of INA preincubation of cells followed by the treatment with fungal elicitor, however, caused the enhancement of PAL activity and mRNA amounts and increased considerably the accumulation of coumarin phytoalexins. Furthermore, the reinforcement of the plant cell wall with ferulic esters was greatly stimulated under these conditions as compared with controls that had not received the INA preincubation (Kauss et al., 1993). PAL can be regarded as an "early bottleneck" enzyme of the stress-inducible phenylpropanoid pathway and it is proposed that INA predisposition of tissues involves the induction of late rather than of early phenylpropanoid enzymes. This kind of induction may provide the key for a much stronger biosynthetic capacity when the early enzymes become induced by subsequent fungal elicitation. This report demonstrated clearly that CCoAOMT and STS are induced in grapevine cells without any visible consequence on incu-

bation with INA. The ferulic cell wall reinforcement is commonly associated with browning of the cells and depends on the hydroxylation of 4-coumaroyl- to caffeoyl-CoA as shown originally in parsley (Kneusel et al., 1989; Pakusch and Matern, 1991; Matern et al., 1995); this pathway has meanwhile been confirmed for many other plants. The hydroxylation is controlled by the cytoplasmic pH, which decreases upon fungal infection or elicitation (Kneusel et al., 1989). Under the premises that rate-limiting early enzymes for phytoalexin synthesis or cell wall reinforcement, e.g. PAL or 4-coumaroyl-CoA hydroxylase, are not induced by INA treatment, the induction of STS or CCoAOMT will not cause immediate effects without subsequent fungal challenge. Such a mechanism is reminiscent of a stretched resilient hunting bow and might explain in part the puzzling phenomenology of the SAR response of grapevine and other plants.

ACKNOWLEDGMENTS

We wish to thank T. Staub, H. Kessmann, and M. Oostendorp (Novartis, Basel, Switzerland) for their continued interest and financial commitment. The STS cDNA (*pSV368*) was kindly supplied by H. Kindl (Marburg, Germany), and *V. vinifera* cv Gamay Fréaux tissue culture was obtained from F. Cormier (St. Hyacinthe, Canada), which is gratefully acknowledged. Oligonucleotides were synthesized by G. Igloi (Biologie III, Freiburg, Germany). We are indebted to B. Grimmig for helpful suggestions and to G. Igloi for critical reading of the manuscript.

Received March 3, 1997; accepted July 26, 1997.

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The GenBank/EMBL accession number of the cDNA sequence of *V. vinifera* CCoAOMT (*V*CCoAOMT) reported in this article is Z54233.

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DNA methylation and plant development

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The development of genetic tools to alter DNA methylation has led to a deeper understanding of the importance of DNA modification in the life strategies of different eukaryotic organisms. This review focuses on recent findings that demonstrate a role for cytosine methylation in the development of higher plants. The effects of altering DNA modification are considered in the context of unique aspects of plant development.

Plant genomes contain substantial amounts of 5-methylcytosine. Up to 20–30% of the cytosines are methylated in the nuclear genome of many flowering plants (angiosperms)[1]. Much of this modification is found in the short symmetrical sites, CpG and CpNpG (Ref. 2), but noncanonical methylation outside of these sites is also found in angiosperm genomes[3]. As in other organisms, methylation of cytosine residues in plants occurs post-replicatively through the action of cytosine-DNA methyltransferases[4]. Plant DNA methyltransferases have been characterized biochemically[5] and plant genes encoding these enzymes have been isolated by similarity to their mammalian counterparts[6].

Investigation of different plant genes and introduced transgenes has found a general, although not invariable, correlation between transcriptional inactivity and increased DNA methylation (reviewed in Refs 7–9), consistent with evidence from mammalian systems. These studies support a role for cytosine methylation in maintaining transcriptional states, but it is unclear whether methylation is used as a primary determinant to establish these transcriptional states. The focus here is to consider specifically the interplay between genomic modification and angiosperm development, an interaction that points to possible functions for DNA modification beyond the reversible control of gene expression during development.

Some important elements of plant development

Consideration of some unique aspects of plant development[10] provides a context in which to understand the effects of manipulating genomic methylation in plants. Most of the structures in a mature plant are built after embryogenesis by the reiterative action of meristems, which are collections of undifferentiated cells poised at the apices of growing roots and shoots. By delaying the bulk of growth and differentiation until after embryogenesis, plants can fashion their form to the environment, allowing development to substitute for behavior in some measure. The identity of the structures produced by the apical meristems changes through developmental time[11]. For example, early in development the shoot apical meristem produces vegetative leaf structures, while later in development a transition to production of reproductive floral structures occurs. The relatively late divergence of vegetative (somatic) and floral (reproductive) lineages allows hereditary alterations that occur through somatic development to be transmitted to subsequent generations.

The need for developmental plasticity and environmental interaction suggests that plants would extensively employ epigenetic regulatory strategies. Such strategies rely on heritable, often reversible, changes in access to the underlying genetic information, but not alteration of the primary nucleotide sequence[12]. Epigenetic strategies might also be elaborated in plants because of the opportunity to select, and to transmit to the next generation, metastable epigenetic states established throughout development in the somatic tissues. Consequently, one should expect the alteration of plant DNA methylation to perturb plant development significantly, provided that differential DNA methylation is an important component of epigenetic regulation in plants. On the other hand, the relative tolerance of aneuploidy and genic imbalance in plants (for example, Ref. 13) predicts that altering DNA modification will have a muted effect on plant development. Work published to date supports both points of view.

DNA methylation changes in plant development

One paradigm linking DNA methylation and development comes from work on the mouse, where average genomic cytosine methylation levels in embryonic lineages drop sharply in the early cleavages following fertilization and rise again around the time of implantation[14] (R. Jaenisch, this issue). This 'erasure-resetting' of genomic methylation in early mammalian development is thought to reflect re-establishment of modification patterns to guide or to reinforce tissue specific gene expression later in development. It is unclear how well the mammalian methylation erasure-resetting paradigm fits plants (see below), because it is difficult to measure cytosine methylation levels in early plant development due to the inaccessibility of very early plant embryos, which are encased in maternal tissue. It is possible, however, to measure DNA methylation content in pollen and post-embryonic tissues of varying age. Information from studies of this type suggests that there is a gradual rise in 5-methylcytosine levels in post-embryonic tissues produced by meristems at positions further from the base of the plant (i.e. tissues of increasing age)[15,16]. Genetic studies of transposon systems in maize also demonstrate an age-dependent gradient of increasing epigenetic modification, which is correlated with DNA methylation[17–19]. Whether age-dependent modification gradients contribute to the control of meristem transitions, or simply reflect meristem age, is one question that has been highlighted by the recent experimental manipulation of genomic methylation.

Methods and consequences of altering DNA methylation in higher plants

Until recently, alteration of DNA methylation in whole eukaryotic organisms was principally achieved by application of DNA methylation inhibitors, such as 5-azacytidine or 5-azadeoxycytidine[20]. Methylation inhibitor studies have induced developmental abnormalities in several plant species, including rice[21], *Triticale*[22], flax[23] and tobacco[24]. Although similar changes were seen in several studies (e.g. dwarfing, reduced fertility), the effects were variable. In some cases, the phenotypes induced in the first generation of exposure to

PII: S0168-9525(97)01199-7

TABLE 1. A partial list of morphological phenotypes associated with genomic hypomethylation in *Arabidopsis thaliana*

Phenotype	Hypomethylation method		
	α-*MET1* CSIRO (Refs 9, 25)	α-*MET1* Yale (Ref. 26)	*ddm1* inbred lines (Ref. 29)
Decreased plant stature	•		•
Reduced apical dominance (bushiness)	•		•
Delayed flowering	•	•	•
Early flowering (vernalization sensitive)	•		
Increased number of inflorescence branches	•	•	
Decreased fertility	•	•	•
Floral abnormalities			
Increased stamen number	•	•	
Homeotic transformations	•		
Unfused carpels		•	•
Curled or twisted leaves	•	•	•

Abbreviation: α-*MET1*, antisense *MET1*.

5-aza(deoxy)cytidine were inherited in subsequent generations[21,23], suggesting the formation of heritable lesions (mutations or epigenetic alterations). In other cases, the induced changes were transitory and were not transmitted in a recognizable manner to progeny. Some of the inhibitor studies also demonstrated inheritance of genomic hypomethylation in progeny[21,22,24]. The inhibitor studies suggest that DNA methylation plays a role in plant development, but it is difficult to separate the nonspecific effects of these agents from DNA methylation effects.

More recently, genetic tools have been developed that allow a more specific and controlled manipulation of genomic methylation levels in plants. A reverse genetic approach has been taken by two different groups[25,26] who made transgenic *Arabidopsis thaliana* plants that express antisense constructs of a native cytosine-DNA methyltransferase gene (*MET1*)[6]. Genomic hypomethylation varies greatly between independent antisense transgenic lines, but the most-severely affected lines have 5-methylcytosine levels ranging from 10 to 30% wild-type content. Single-copy and repetitive DNA sequences are hypomethylated in the *MET1* antisense lines.

Taking a traditional genetic approach, my group has isolated *A. thaliana* mutations (*ddm*, decrease in DNA methylation) that reduce nuclear 5-methylcytosine levels[27]. The best-characterized mutations define the *DDM1* gene. Homozygotes carrying recessive *ddm1* alleles contain 30% of the wild-type levels of 5-methylcytosine. The *ddm1* mutations do not map to the two known cytosine-DNA methyltransferase genes of *A. thaliana*[6], nor do they affect DNA methyltransferase activity detectable in nuclear extracts[28]. In addition, *ddm1* mutations do not appear to affect the metabolism of the active methyl group donor, S-adenosylmethionine[28]. Consequently, the *DDM1* gene product is a candidate for a novel component of the DNA methylation system, or is involved in determining the cellular context (e.g. chromatin structure, subnuclear localization) of the methylation reaction. The *ddm1* mutations affect the single-copy and repetitive component of the genome to different extents and with different kinetics[29]. Strong hypomethylation of repetitive DNA families is seen in *ddm1* homozygotes from segregating families, but hypomethylation of single-copy sequences is progressive and usually detected only after the mutants have been inbred for several generations[29].

FIGURE 1. An example of a morphological defect developed in an inbred *ddm1* mutant line. The plant on the left has a normal phenotype, while the plant on the right has the 'bal' phenotype, characterized by twisted leaves and small plant stature. The plants are 34-day-old siblings from a population segregating a heritable *bal* lesion developed in an inbred *ddm1* mutant (Fig. 2). In this example, the *ddm1* mutation has been removed and the genotype of the parent of the plants shown was *DDM1/DDM1; BAL/bal*.

The phenotypic consequences of lowering genomic methylation in *A. thaliana* through genetic means are varied, but striking (Table 1; Fig. 1). A variety of morphological phenotypes arose in the *MET1* antisense lines and different lines had different combinations of phenotypic characters[25,26]. Similarly, independent inbred *ddm1* mutant lines had different constellations of developmental defects[29]. Related phenotypic characters occur in the different *ddm1* and *MET1* antisense lines, indicating that DNA methylation plays a role in the regulation of processes controlling these characters. The data also present some contradictions, in particular, the apparently divergent effects on flowering time. This discrepancy might be explained by the different environmental conditions and genetic backgrounds used in the experiments. For example, the CSIRO group transformed an *A. thaliana* strain, C24, that is sensitive to vernalization (cold-treatment induction of flowering). *MET1* antisense expression in C24 leads to earlier flowering[9], consistent with reports that 5-azacytidine application can substitute for vernalization[30,31]. On the other hand, the Yale *MET1* antisense lines and the *ddm1* mutants are in the vernalization-insensitive Columbia background. *MET1*

antisense expression in the C24 lines does not completely substitute for vernalization[9] and the resulting intermediate flowering time might reflect a superimposition of a delay in flowering and removal of the vernalization requirement.

An association between developmental defects and DNA hypomethylation was shown in a number of ways. In the antisense expression studies[25,26], the severity of the morphological abnormalities is correlated with the extent of genome-wide hypomethylation. In addition, removal of the *MET1* antisense construct by genetic segregation leads to genomic remethylation and a reduction in the severity of the morphological phenotypes[26]. In the *ddm1* mutant studies[29], progressively more severe developmental defects were seen upon inbreeding the mutants, which coincides with the progressive loss of DNA methylation in the single-copy genomic component. Isogenic *DDM1* wild-type control lines fail to show phenotypic defects or methylation changes, demonstrating that this inbreeding depression is dependent on the DNA hypomethylation background (Fig. 2). The parallels between the effects caused by *ddm1* and *MET1* antisense lines indicate that DNA hypomethylation is directly involved in the onset of phenotypic abnormalities in *ddm1* lines.

In contrast to the *MET1* antisense results, removal of the hypomethylation locus does not lead to reversion of the morphological phenotypes that develop in *ddm1* inbred lines. Genetic analysis indicates that different phenotypic characters in *ddm1* inbred lines can be separated from the potentiating *ddm1* mutation and mapped to heritable lesions at dispersed sites in the genome[29] (Fig. 2). In the cases examined to date, the lesions behave as stable mendelian factors, some of which act as dominant alleles and others as recessive alleles[29] (T. Kakutani, pers. commun.). Lesion formation appears to be targeted to particular loci of the *A. thaliana* genome as evidenced by the recovery of a number of independent dominant lesions that condition late flowering and map to the same genetic location (T. Kakutani, pers. commun.).

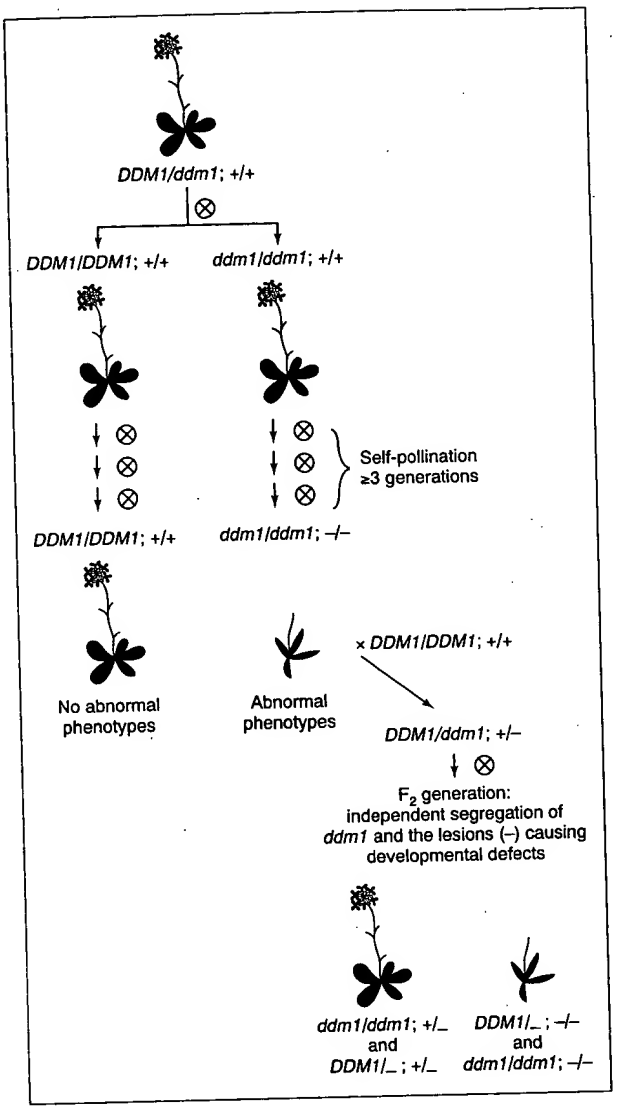

FIGURE 2. Developmental defects in inbred *ddm1* lines are caused by unlinked heritable lesions. A schematic pedigree illustrating the formation of heritable lesions (designated by '–', where '+' is the wild type and '_' is unknown/unspecified) in inbred (⊗) *ddm1* mutant lines. Abnormal phenotypes develop in *ddm1/ddm1* inbred lines but not in inbred *DDM1/DDM1* sibling lines. Outcrossing phenotypic *ddm1/ddm1* plants to wild-type individuals generates F_1 plants that give rise to F_2 populations independently segregating the *ddm1* mutation and the lesions causing the developmental defects.

Mechanisms responsible for DNA hypomethylation-induced developmental defects in plants

It is tempting to ascribe the development defects to aberrant gene expression caused by DNA hypomethylation. Indeed, ectopic expression of two floral homeotic genes controlling organ identity (*AG* and *AP3*) occurs in the *MET1* antisense plants examined by the CSIRO group[25]. Such abnormal gene expression patterns are likely to underlie the floral homeotic transformations and leaf curling phenotype. The recent finding that ectopic *AG* expression also occurs in the *A. thaliana curly leaf* mutant, which contains a defective Polycomb-group protein[32], has led to the suggestion that DNA methylation and higher-order chromatin organization collaborate to cement epigenetic gene expression states through development[25]. A variant model proposes that DNA hypomethylation of transposable elements, which are known to incorporate differential DNA methylation as an epigenetic regulation mechanism, leads to abnormal expression of nearby genes[33,34]. In no case has the molecular structure of genes aberrantly expressed in the hypomethylation backgrounds been examined to determine whether or not loss of methylated sites in the vicinity of the gene contributes to misexpression.

The late-flowering phenotype seen in the Yale *MET1* antisense lines might involve mechanisms that operate above the level of individual genes. The delayed flowering and increased inflorescence (flowering stem) branch number seen in the *MET1* antisense lines are consistent with a heterochronic shift towards juvenility (that is the phase change from juvenile to adult development is delayed). Ronemus *et al.*[26] suggest that the heterochrony might stem from a dampening of the increasing DNA methylation gradient normally seen in progressively older tissues[11]. In this view, the DNA methylation gradient serves as a developmental clock that runs more slowly

than normal in the hypomethylation background. The clock model does not exclude the idea of gene misexpression because it is possible that meristem transitions are controlled by genes whose expression is graded by a progressive methylation shift through plant development.

A different perspective on the role of DNA methylation is provided by the *ddm1* mutant work[29]. The specificity and heritability of *ddm1*-induced lesions, and the high frequency of lesion formation, highlight the importance of DNA methylation in the maintenance of genome integrity. The molecular nature of the lesions is currently under investigation but, regardless of the mechanism at work, it is clear that DNA hypomethylation allows the accumulation of heritable alterations throughout the genome. The lesions might be genetic alterations (e.g. point mutation, transposon insertion, DNA rearrangement), but many characteristics of the onset of *ddm1*-induced lesions (e.g. progressivity, high frequency, specificity) suggest that the alterations are likely to be epigenetic in origin. In this model, epigenetic states normally cemented or reinforced by DNA methylation are free to shift in *ddm1* backgrounds. The progressive severity of some phenotypes in advancing generations of *ddm1* inbred lines suggests that the proposed epigenetic shifts are not fluctuating but are unidirectional, leading to increasingly severe characteristic phenotypes, rather than to phenotypic variegation.

Because *ddm1* leads to progressive hypomethylation of single-copy sequences during inbreeding, the simplest explanation is that the lesions are epimutations (heritable loss of methylated sites)[12]. The progression towards the characteristic phenotypes is proposed to reflect continual generation of more-severely hypomethylated haplotypes and segregation of these newly formed haplotypes in selfed progeny. Previous experiments[27] have demonstrated that hypomethylated loci can be inherited through meiosis in plants, suggesting that the mammalian model of resetting the methylation pattern in early development does not apply in plants. In principle, therefore, demethylation events could lead to heritable lesions in plants.

Why would *ddm1*-induced epimutations be more stable than *MET1* antisense hypomethylation events? One possibility is that *ddm1*-induced hypomethylation is quantitatively or qualitatively different from the hypomethylation conditioned by *MET1* antisense expression, and leads to failure of the *de novo* methylation machinery to recognize *ddm1* hypomethylated loci. Support for this view comes from the different degrees of remethylation seen after transmission of hypomethylated genomes into a wild-type background. Substantial genome remethylation can be detected in the first-generation segregants lacking the *MET1* antisense transgene[25,26], but hypomethylated chromosomes inherited from a *ddm1* mutant are not appreciably remethylated in wild-type *DDM1* backgrounds[27].

Synthesis and implications

Despite suffering significant developmental defects, *A. thaliana* plants with a 60–90% reduction in DNA methylation are viable and many can still complete development to produce seed. In marked contrast stands the embryonic-lethality of mice containing comparable reductions in genomic 5-methylcytosine[35]. One interpretation is that higher plants and mammals use DNA methylation in fundamentally different ways. A more likely explanation lies in the differences between plant and animal development. The development plasticity of plants and their relative tolerance for perturbation of gene expression might allow execution and completion of the plant developmental program, despite gross alteration of genomic modification. The role of parental imprinting in plant and mammalian development is another probable factor. Mice have a strict requirement for proper imprinting of parental genomes for completion of development[36] (R. Jaenisch, this issue) and mutant studies have demonstrated that DNA methylation is essential for maintenance of the differentially imprinted states in the mouse[37,38]. Imprinting in maize appears to affect mainly the endosperm, which is a triploid (two maternal genomes:one paternal genome) nutritional support tissue in the seed, and proper development of plant embryos does not require inheritance of differentially imprinted genomes[39].

Now that the effects of plant genome modification defects are beginning to be understood at the organismal level, a more targeted approach towards understanding the functions of DNA methylation can proceed. Part of this approach will be the determination of how abnormal methylation of particular genes leads to developmental phenotypes. In addition, the possibility that DNA methylation modulates processes that act above the level of individual genes should receive particular attention. The availability of genetic tools to manipulate DNA methylation and the viability of organisms with grossly altered genomic modification make plants attractive model organisms for studying the functions of eukaryotic DNA methylation.

Acknowledgements

I apologize to authors whose work was not cited because of space constraints. I thank my colleagues for critical input and the National Science Foundation for support. I also thank E.J. Finnegan, T. Kakutani, R. Martienssen and S. Dellaporta for input, specific suggestions on the manuscript, and permission to cite unpublished data.

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DNA methylation and imprinting: why bother?

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DNA methylation is crucial for mammalian development because embryos that cannot maintain normal methylation levels die after gastrulation. I propose that DNA methylation is only important for the somatic lineages, but has no role in embryonic lineages including the germ line. Among vertebrates, genomic imprinting is found only in mammals, and numerous hypotheses have ascribed an essential function to imprinting because of the uniquely mammalian developmental and physiological requirements. However, our understanding of molecular details of the imprinting process, as well as evolutionary considerations, is rather consistent with imprinting having no intrinsic role in mammalian development.

Mammalian development requires genetic contribution from the mother and the father, and the two alleles of most parental genes are functionally equivalent. However, as originally shown by genetic and embryonic manipulations[1,2], the two sets of some parental chromosomes carry imprinted genes that serve differential and, possibly, complementary functions during mammalian development. Imprinted genes are defined as those genes whose expression is determined by their parental origin (see Table 1 for definition of the genes discussed in this article). For example, in mice and humans the imprinted *Igf2* gene is always expressed from the paternal, but not from the maternal, chromosome[3–6]. Disturbance of normal imprinting, as seen in patients with uniparental disomy, can lead to biallelic expression of imprinted genes and can result in severe developmental abnormalities or cancer[7,8].

To understand the process of imprinting we have to explain how the imprinting marks are established, how the marks are read and maintained in the somatic cells of the adult, and how they are erased during gametogenesis to reverse silencing. DNA methylation represents an attractive mechanism of 'genetic memory' because this epigenetic mark is clonally inherited and, thus, can distinguish the two alleles of imprinted genes. Indeed, the inheritance of methylation patterns imposed on alleles of imprinted genes is entirely consistent with methylation being a crucial player in the maintenance and establishment of imprints. The role of methylation in development, and the evidence for a causative link between methylation and imprinting, is summarized in the first part of this review. Imprinting is a uniquely mammalian phenomenon in search of a reason, and many different hypotheses have been proposed to rationalize its role in development, and to define the evolutionary pressure that led to its emergence and fixation in mammalian populations. In the second part of the review I discuss the evidence that imprinting is a by-product of mammalian evolution due to the unique relationship between mother and embryo.

Methylation and development

The methylation level characteristic for the mammalian genome is established around gastrulation (Fig. 1). Before gastrulation, a highly regulated process first erases

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Publisher: Rockville, MD : American Society of Plant Physiologists, 1926-
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CS Dep. Biology, Campus Box 1137, Washington Univ., One Brookings Drive, St. Louis, MO 63130 USA
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AU van der Wilk, Frank; Houterman, Petra; Molthoff, Jos; Hans, Fabienne; Dekker, Ben; van den Heuvel, Johannes; Huttinga, Harm; Goldbach, Rob
CS DLO Res. Inst. Plant Protection (IPO-DLO), Wageningen, 6700 GW, Neth.
SO Molecular Plant-Microbe Interactions (1997), 10(2), 153-159
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CS Dep. Biol., Washington Univ., St. Louis, MO, 63130, USA
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CS Department of Biology, Yale University, New Haven, CT 06520-8104, USA.
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VOLUME 10, NUMBER 2 • MARCH 1997 • ISSN 0894-0282

MPMI Vol. 10, No. 2, 1997, pp. 153–159. Publication no. M-1997-0113-01R.

Expression of the Potato Leafroll Virus ORF0 Induces Viral-Disease-like Symptoms in Transgenic Potato Plants

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Received 30 July 1996. Accepted 4 December 1996.

The role of the open reading frame 0 (ORF0) of luteoviruses in the viral infection cycle has not been resolved, although the translation product (p28) of this ORF has been suggested to play a role in host recognition. To investigate the function of the potato leafroll luteovirus (PLRV) p28 protein, transgenic potato plants were produced containing the ORF0. In the lines in which the ORF0 transcripts could be detected by Northern (RNA) analysis, the plants displayed an altered phenotype resembling virus-infected plants. A positive correlation was observed between levels of accumulation of the transgenic transcripts and severity of the phenotypic aberrations observed. In contrast, potato plants transformed with a modified, untranslatable ORF0 sequence were phenotypically indistinguishable from wild-type control plants. These results suggest that the p28 protein is involved in viral symptom expression. Southern blot analysis showed that the transgenic plants that accumulated low levels of ORF0 transcripts detectable only by reverse transcription–polymerase chain reaction, contained methylated ORF0 DNA sequences, indicating down-regulation of the transgene provoked by the putatively unfavorable effects p28 causes in the plant cell.

The genome of potato leafroll virus (PLRV), a species of the genus *Luteovirus*, consists of a single-stranded, messenger-sense RNA molecule comprising six open reading frames (ORFs) (van der Wilk et al. 1989; Mayo et al. 1989; Keese et al. 1990). The genomic RNA contains a VPg and is encapsidated in an icosahedral particle. The viral capsid is composed of two proteins. The major coat protein is encoded by the ORF3 present in the 3′-half of the genomic RNA. A minor readthrough protein is expressed by translational readthrough of the ORF3 and is believed to be an important aphid transmission determinant of the virus (Brault et al. 1995; Chay et al. 1996; Ziegler-Graff et al. 1996).

Currently, little is known about the role of most of the products of the nonstructural genes of luteoviruses. The ORF2 represents the putative viral polymerase gene, since its product contains the GxxxTxxxN(x_{25-40}) GDD amino acid sequence motif, which is conserved in all known RNA-dependent RNA polymerases (Koonin 1991). The ORF1 product contains a protease motif (Bazan and Fletterick 1989; Gorbalenya et al. 1989), but the role of this putative protease in the viral infection cycle is still concealed. The ORF4 product most probably constitutes the viral movement protein, since it has been shown to bind single-stranded nucleic acids (Tacke et al. 1991, 1993) and to be indispensable for systemic infection of plants (Chay et al. 1996). The function of the ORF0 product is still obscure. ORF0 encodes a 28-kDa protein that is highly hydrophobic and shows a weak homology with several membrane-linked proteins (Mayo et al. 1989).

Analysis of the amino acid sequence of PLRV ORF0 has revealed a putative membrane-binding site between residues 21 and 32 (Mayo et al. 1989). The genomic organization of PLRV is very similar to those of the other subgroup II luteoviruses, e.g., beet western yellows virus (BWYV) (Veidt et al. 1988). Comparisons made between the PLRV- and BWYV-encoded proteins revealed that all the viral proteins share a high homology in amino acid sequence except for the ORF0s. Although the ORF0s of both viruses are similar in size and position on the genome, the primary structures of their respective products (p28) are not recognizably similar. Furthermore, there are no indications that the BWYV ORF0 product is membrane-linked. From an ecological point of view the main difference between the two viruses lies in their host ranges, PLRV being only capable of infecting a limited number of plant species (mostly *Solanaceae*) (Harrison 1984) and BWYV able to infect many different plant species (Duffus 1972). Since the ORF0 products seem to constitute the main genetic difference between the viruses, it has been suggested that ORF0 plays a role in host recognition (Veidt et al. 1992).

The transformation of plants with viral nonstructural genes is a useful approach to the analysis of an interaction between the virus-encoded proteins and the host plant. To investigate the function of PLRV p28, potato plants were transformed with the ORF0. Plants expressing this ORF0 displayed a viral-disease-like phenotype, indicating that p28 interferes with the metabolism of the plant.

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RESULTS

Analysis of transgenic potato plants expressing the PLRV ORF0.

Transgenic potato plants, cv. Désirée, were produced harboring the PLRV ORF0. To this end, a cDNA fragment consisting of the wild-type ORF0 sequence, including the 5′ end of the underlying ORF2, was cloned into a binary transformation vector (Fig. 1A). Transformation of tuber disks was mediated by *Agrobacterium tumefaciens*. Forty-four independent transformants were obtained.

Eighteen of the produced plant lines were phenotypically dissimilar from the other transformants or from nontransgenic potato plants. Five of these plant lines displayed stunting and deformation of the leaves; these types of aberrations have been observed previously in potato transformations and were probably caused by somaclonal variation during the regeneration process. The other deviant plants displayed yellowing and rolling of the leaves, anthocyan formation in the leaf, and slow and (severely) stunted growth of the plant (Fig. 2). The leaves of these plants were thickened and brittle. The plants displaying the more extreme phenotypic aberrations exhibited slow or impeded root formation both in soil and on medium containing or devoid of kanamycin. Several of the obtained transgenic plants were not able to produce viable tubers. In general, the observed phenotypic aberrations strongly resembled viral-disease-like symptoms. The changes in the phenotypic appearance of the plants were not due to conditions in tissue culture, since plants grown from tubers displayed exactly the same phenotype. The disease-like symptoms developed approximately 7 days after planting or within 3 days after sprouting, and increased with aging of the plants.

The manifestation and extension of the phenotypic aberrations were strongly influenced by the light intensity used to grow the plants. High light intensities induced strong phenotypic aberrations, while at low light intensities phenotypic changes were milder. This is analogous to symptom development of luteovirus-infected plants, in which symptoms are enhanced by elevated light intensities (Rochow 1970; Duffus 1972; Ashby 1984).

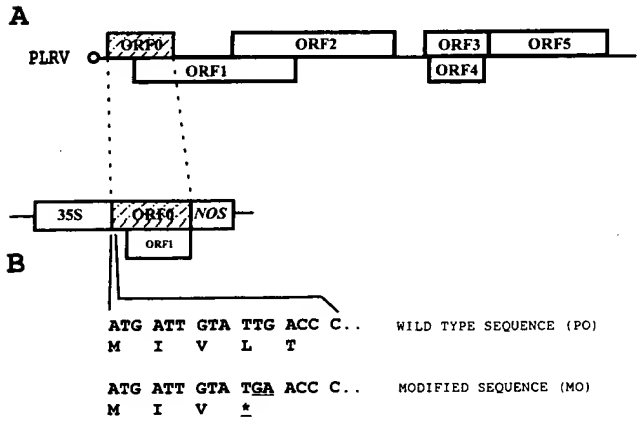

Fig. 1. Schematic representation of the potato leafroll virus (PLRV) genome (**A**) and construction of the open reading frame 0 (ORF0) plant transformation vectors (**B**); 35S = cauliflower mosaic virus (CaMV) 35S promoter; *nos* = nopaline synthase terminator. PO: wild-type ORF0 sequence; MO: modified ORF0 sequence. Modifications are underlined.

The RNA in the transgenic plants was tested by Northern (RNA) blotting to detect the presence of ORF0-specific mRNA (Fig. 3). In 14 of the transgenic plants there were detectable levels of ORF0 mRNA. With the exception of one plant line (PO34), the presence of detectable amounts of ORF0 mRNA in the transgenic potato plants coincided with the presence of virus-like symptoms. This line (PO34) was indistinguishable from nontransgenic potato plants but contained detectable levels of ORF0 mRNA. In all other cases, the severity of the virus-like symptoms was directly correlated with the levels of accumulation of ORF0 transcripts, suggesting that expression of PLRV ORF0 sequences caused the appearance of symptoms similar to those of a PLRV-infected plant.

To determine whether accumulation of ORF0 mRNA had an effect on host mRNA accumulation, the transgenic plants were tested for accumulation of mRNA encoding actin and the small sub-unit of ribulose-biphosphate-carboxylase (rubisco). This analysis revealed that all lines contained comparable amounts of mRNA coding for actin (data not shown), but that the accumulation of rubisco mRNA was strongly inhibited in the plants displaying virus-like symptoms (Fig. 3). In plants showing extreme phenotypic aberrations, accumulation of rubisco mRNA was barely detectable by Northern analysis. These plants were extremely stunted, and the leaves were distorted and ranged in color from yellow to brown.

To verify whether the transgenic plant lines displaying the altered phenotype contained complete, nonmutated ORF0 sequences, polymerase chain reaction (PCR) was carried out with isolated genomic DNA as a template. The 5′-terminal primer used in the PCR experiments was identical to a sequence internally located in the cauliflower mosaic virus (CaMV) 35S promoter, while the downstream primer was complementary to the 3′ end of ORF0. PCR performed with genomic DNA isolated from all lines, including those that did and those that did not express ORF0 mRNA, produced DNA fragments of the expected size. Sequence analysis of these PCR products showed that all plants tested contained unaltered copies of the PLRV ORF0 sequence.

Analysis of transgenic and PLRV-infected potato plants for p28.

To detect p28, all the transgenic plants and PLRV-infected potato plants were tested in Western blot procedures or in enzyme-linked immunosorbent assays (ELISAs). In the Western blot analysis an antiserum raised against an *E. coli* expressed p28/GST fusion protein was used and in ELISA antisera were used that had been raised against synthetic peptides. The latter antisera had been shown previously in immunofluorescence experiments to recognize native p28 protein expressed in SF9 insect cells, using the baculovirus expression system (data not shown). The detection limit of the antiserum used in the Western blot procedure was approximately 1 ng of purified GST-p28 protein, while the other antisera exhibited a slightly lower affinity. Since it has been suggested that p28 is membrane-associated (Mayo et al. 1989), several different procedures were examined to isolate p28 from transgenic or infected plant tissue, including methods specifically designed to isolate membrane-linked proteins (Deom et al. 1990; Martin and Garcia 1991). However, in transgenic and infected plants p28 protein could be detected neither on Western blot nor by ELISA.

Production and characterization of transgenic plants harboring a modified ORF0 sequence.

Since it was not possible to detect the p28 protein in the transgenic plants it was investigated whether translational expression of PLRV ORF0 initiated the observed viral-disease-like symptoms. The alternative explanations were that the phenotypic aberrations were caused by the transformation procedure, the underlying 5′-terminal sequence of the ORF2, or the presence of ORF0 RNA sequences themselves. To this end, potato plants (cv. Désirée) were transformed with a modified ORF0 sequence (MO) or with the wild-type sequence (PO) as a control. As shown in Figure 1B, the modified se-

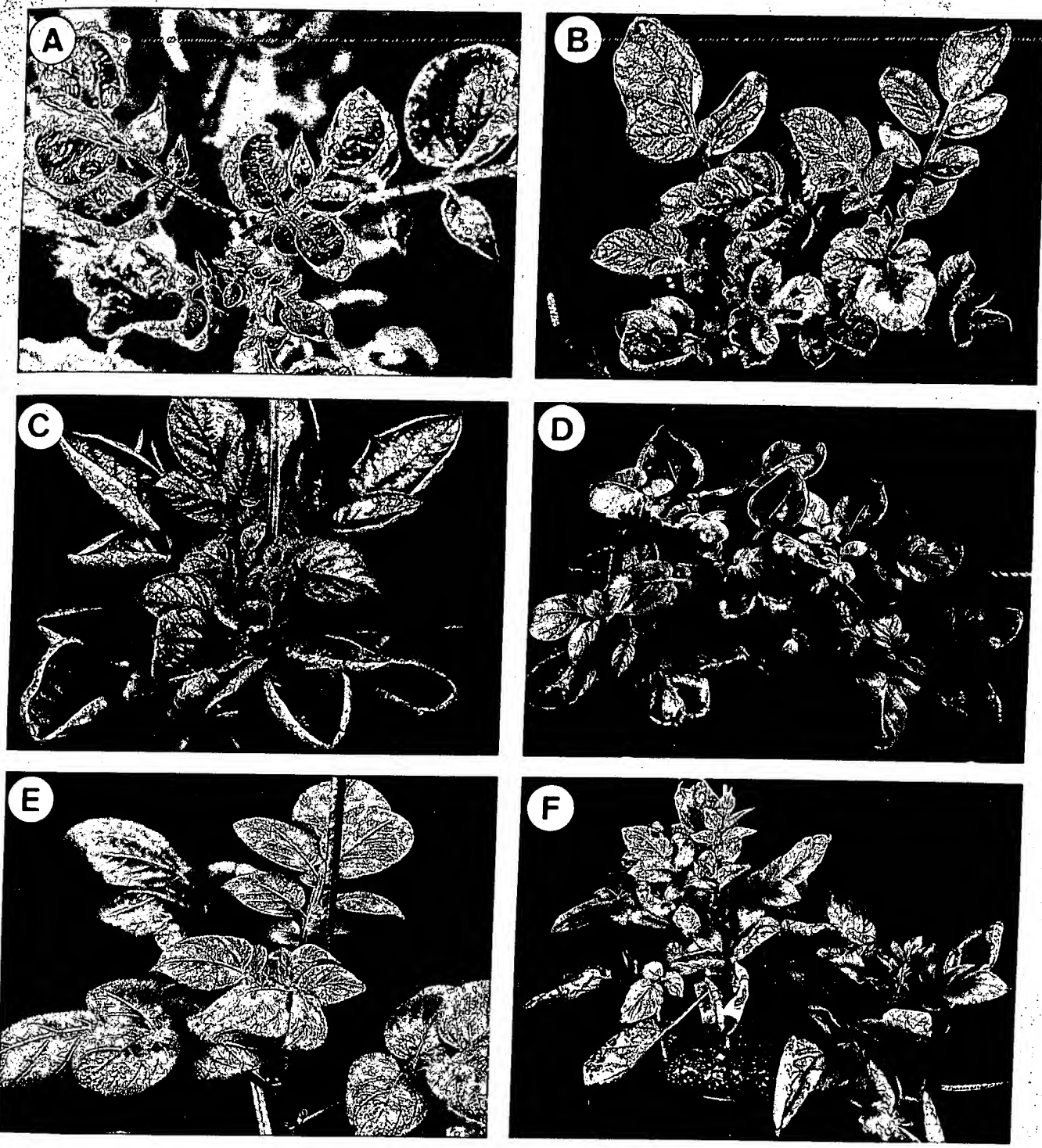

Fig. 2. Phenotypes of potato leafroll virus open reading frame 0 (PLRV ORF0) transgenic potato plants expressing detectable levels of transgenic transcripts. **A, B, C,** and **D,** transgenic lines PO4, PO18, PO24, PO134, respectively. **E,** Wild-type potato plant cv. Désirée. **F,** Cv. Désirée infected with PLRV.

quence contained a stop codon (UGA) 6 nucleotides downstream of the start codon, thus blocking the translation of the ORF0 reading frame. The next possible start for translation of the p28 is located at Met^{144}, virtually excluding the possible generation of a truncated p28 protein.

The MO construct was introduced into 80 independent lines and the PO construct into 25 lines. Regeneration of the MO plants in general took 3 to 9 weeks less than that of the PO plants; all MO lines but 14 were phenotypically similar to wild-type potato. These 14 MO lines displayed stunting and distortion of the leaves. Rolling and yellowing of the leaves and anthocyan formation were never observed. The phenotypic aberration of these MO lines was similar to the phenotype associated with somaclonal variation. Northern blot analysis of 80 transgenic MO plant lines disclosed that 90% of the examined plants contained detectable levels of ORF0 transcripts.

The results from the transformation experiments with the wild-type ORF0 sequence were comparable to the results from the initial transformation experiment. In total, 25 independent transgenic plant lines were obtained. Only five plant lines accumulated full-length ORF0 transcripts detectable on Northern blot. Two of these plant lines showed severe phenotypic aberrations probably caused by somaclonal variation occurring during the regeneration process. However, two transgenic plant lines displayed the typical viral-disease-like symptoms identical to the earlier observed phenotype.

Southern blot analysis of the transgenic plants.

The results from the aforementioned experiments indicate that the accumulation of ORF0 transcripts was suppressed in the PO plants. Methylation of DNA sequences has been shown to play a role in reduction of the transcription of transgenes (for review see Razin and Cedar 1991). Therefore, Southern blot analysis was carried out to determine whether methylation of the inserted ORF0 sequences played a role in down-regulation of the transcription of the inserted ORF0 construct. To this end, genomic DNA was digested with *Hin*dIII and *Pst*I. The restriction endonuclease *Pst*I is inhibited by the presence of an N^6-methyladenine or 5-methylcytosine residue in its recognition site. *Pst*I restriction sites are present both in the 5′-terminal sequence of the ORF0 and in the bordering T-DNA upstream of the 35S promoter still inserted in the genome of the plant, while *Hin*dIII cleaves immediately after the *nos* terminator. Digestion of the plant genomic DNA with *Hin*dIII and *Pst*I would therefore result in the occurrence of two fragments of approximately 1,300 (*Pst*I/*Hin*dIII) and 850 base pairs (*Pst*I/*Pst*I), unless one of the restriction sites was blocked. As shown in Figure 4, both expected fragments could be detected in the lanes loaded with *Pst*I/*Hin*dIII-digested DNA derived from plants expressing detectable levels of ORF0 transcripts. Non-expressors either did not contain a full-length copy of the ORF0 or a fragment was revealed of approximately 2.2 kb. A fragment of this size could only arise by non-digestion of the ORF0 internal *Pst*I site. However, interpretation of the results was complicated by the presence of smaller fragments possibly caused by deletion of transgene sequences. To verify whether the occurrence of the 2-kb fragment was caused by specific blocking of the ORF0 *Pst*I site and was not due to overall incomplete digestion of the genomic DNAs, the blots were hybridized with a *Pst*I-derived rubisco fragment. Since in all lanes only fragments of the expected sizes (approximately 2 kbp) were observed, it was concluded that all DNAs were completely digested. From these data it was deduced that the transgene ORF0 sequences were methylated. The copy numbers of the integrated ORF0 constructs, as determined by Southern analysis, were estimated to be between one and three (data not shown).

To determine whether methylation of the transgene sequences completely inhibited transcription, reverse transcription PCR was carried out with primers identical to the 5′ end

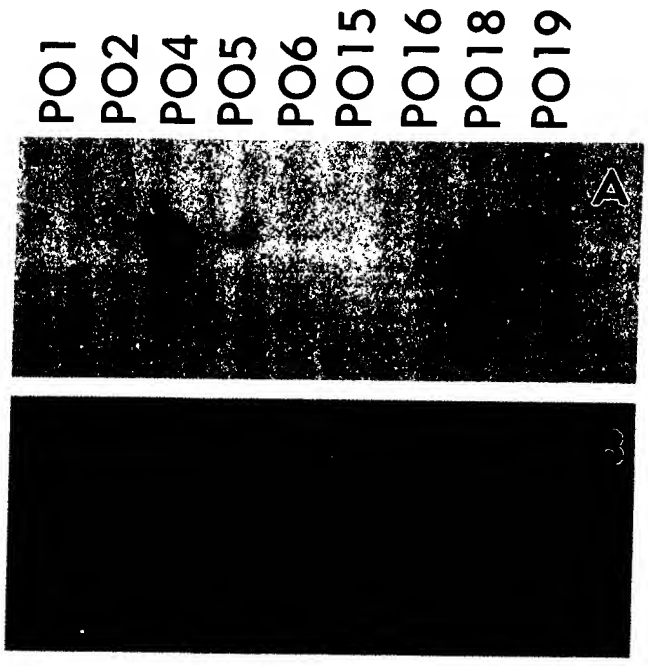

Fig. 3. Northern (RNA) blot analysis of potato leafroll virus open reading frame 0 (PLRV ORF0) transgenic potato plants. Aliquots (20 μg) of total plant RNA purified from transgenic leaf material were loaded onto a 1% agarose gel containing formaldehyde. **A,** Blot probed with a radiolabeled ORF0 DNA fragment. **B,** Blot probed with radiolabeled rubisco DNA.

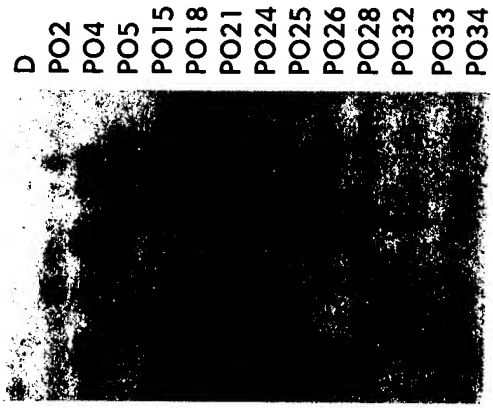

Fig. 4. Southern blot analysis of transgenic potato plants. Aliquots of (40 μg) of DNA, purified from transgenic leaf material, and digested with *Pst*I and *Hin*dII,I were loaded onto a 1% agarose gel and subsequently probed with a radiolabeled open reading frame 0 (ORF0) DNA fragment.

and complementary to the 3′ end of the ORF0. All transgenic plants shown to contain full-length copies by Southern analysis yielded ORF0-related PCR products, indicating that low levels of transcripts were present and that methylation did not completely inhibit transcription.

DISCUSSION

Except for the structural proteins, little is known about the functions of the putative products of the ORFs present on the genome of PLRV in particular and of luteoviruses in general. It is still obscure which ORF encodes the VPg, and concerning the nonstructural genes only the putative RNA-dependent RNA polymerase (by sequence comparison) and a putative movement protein have been identified (Tacke et al. 1991, 1993; Chay et al. 1996). To gain insight in the function of the non-structural ORF0 of PLRV, we have transformed potato with this sequence. The resulting transgenic (PO) plants that accumulated levels of ORF0 transcripts detectable on Northern blot displayed a phenotype resembling viral-diseased plants. Since transgenic MO plants that expressed a modified, untranslatable ORF0 sequence were phenotypically identical to wild-type potato plants, it is concluded that translational expression of the PLRV ORF0 provoked the observed viral-disease-like symptoms.

Northern blot analysis of the transgenic PO plants revealed that accumulation of mRNA encoding rubisco was impeded. Both the change in phenotype and the decrease in accumulation of rubisco mRNA indicate that expression of the ORF0 interfered with the metabolism in the plant cells. High light intensities used to grow the plants ameliorated the severity of the observed symptom-like phenotype, suggesting that p28 intervenes in the photosynthesis. An alternative explanation is that p28 induces blocking of the transport through the floem, resulting in accumulation of high levels of metabolites in the cell and subsequent suppression of photosynthesis.

Attempts to detect the ORF0 product in transgenic tissues were unsuccessful. p28 has been suggested to be membrane-linked (Mayo et al. 1989). However, with procedures reported to be successful for the isolation of viral membrane-bound proteins, p28 could not be detected in either ELISAs or Western blots. These findings coincide with our findings that the detection of p28 is infeasible in PLRV-infected plant tissues and protoplasts (data not shown). Possibly, p28 is rapidly degraded in the plant cell to circumvent a toxic effect imparted by the accumulation of p28 in the cell.

Approximately one third of the obtained transgenic PO plants accumulated levels of ORF0 mRNA detectable in Northern blot procedures. In contrast, 90% of the MO transgenic potato plants containing the modified, untranslatable ORF0 sequence were shown to accumulate detectable levels of transgenic transcripts. Furthermore, the level of accumulation of transgenic mRNA in plants expressing the wild-type ORF0 was notably lower than in transgenic plants expressing the modified ORF0 sequence. In all experiments, the same binary transformation vector, and thus promoter, was used to express the different ORF0 sequences in plants. Differences in accumulation of mRNA have been shown to be caused by variations in the level of transcription due to methylation of transgenic sequences (for review see Razin and Cedar 1991) or by post-transcriptional degradation of the mRNA (van Blokland et al. 1994). Southern blot analysis revealed that the plants accumulating low levels of transgenic transcripts contained methylated ORF0 sequences, while in plants expressing high levels of ORF0 transcripts non-methylated transgene DNA was predominant. It has been reported that gene inactivation by methylation is influenced by the copy number of the transferred gene (Linn et al. 1990; Scheid et al. 1991). However, we have not observed a correlation between the estimated transgene copy numbers and methylation. Possibly, the observed suppression of transgene expression by methylation was triggered by the putatively disadvantageous effects p28 causes in the cell.

Previously, it has been reported that expression of the BL1 protein of the geminivirus squash leaf curl virus (SqLCV) (Pascal et al. 1993), the p19 and p22 protein of tomato bushy stunt virus (TBSV) (Scholthof et al. 1995a), and the gene VI product of the pararetrovirus CaMV (Baughman et al. 1988) induced viral-disease-like phenotypes. While expression of CaMV gene VI conveyed viral-disease-like symptoms in non-host plants but failed to do so in susceptible host plants (Goldberg et al. 1989), expression of SqLCV BL1 and TBSV p19 induced viral-disease-like symptoms in their permissive host, *Nicotiana benthamiana*. SqLCV is a bipartite, phloem-limited, circular, single-stranded DNA virus, while TBSV is a single-stranded RNA virus. Interestingly, both BL1 and p19 have been identified as movement proteins and have been shown to be important determinants of viral host range properties (Lazarowitz 1991; Ingham and Lazarowitz 1993; Scholthof et al. 1995b), and both SqLCV and TBSV encode two proteins involved in transport of the virus, BR1/ BL1 and p19/ p22, respectively. The function of the PLRV p28 remains an enigma, but it may well be that the protein is involved in movement of the virus in conjunction with the ORF5-encoded, 17-kDa protein, analogous to the situation of TBSV and SqLCV.

MATERIALS AND METHODS

Production of transgenic plants.

The sequence encoding the PLRV ORF0 was excised from an existing cDNA clone (van der Wilk et al. 1989) employing the PCR. Two ORF0 constructs with different oligonucleotides were synthesized. The first construct (pORF0) was analogous to the wild-type PLRV ORF0 sequence, while the second one (pORFM) contained a stop codon 6 nucleotides downstream of the ORF0 start codon, thus inhibiting translation (Fig. 1B). To facilitate further subcloning, the ORF0 sequences were supplied with *Bam*HI restriction sites located immediately in front of and after the start and stop codons. To exclude the occurrence of possible mutations or deletions the obtained fragments were translated in vitro with the TnT T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI) and sequenced. Subsequently, the fragments were placed between the CaMV 35S-promoter and the nopaline synthase terminator (*nos*) by ligation in *Bam*HI-digested pMOG181. The resulting plasmids (pMOGORF0 and pMOGORFM) were digested with *Eco*RI and *Hin*dIII and the fragments containing the ORF0 cassette were inserted in the binary vector pMOG402, giving rise to the plasmids pBPO and pBMO. Transformation of potato plants was done as previously described (Hoekema et al. 1989). Potato tuber disks (cv. Dési-

rée) were co-cultivated with *Agrobacterium tumefaciens* LBA4404 harboring pBPO or pBMO. For co-cultivation, 1,000 tuber disks were used in the first experiment and 500 discs in the second. Selection for transformation was done on medium containing kanamycin (100 µg/ml). Kanamycin resistant shoots were cut off and placed onto rooting medium, propagated axenically, and transferred to soil.

Analysis of RNA in transgenic potato plants.

The transgenic plants were analyzed on Northern blot for expression of ORF0 encoding mRNA. Total RNA was isolated from plant material following the procedure described by Verwoerd et al. (1989). Approximately 20-µg aliquots of total RNA were loaded onto a denaturing 1% agarose gel containing 2.2 M formaldehyde. After electrophoresis the RNA was transferred to a blotting membrane (Hybond-N, Amersham, U.K.) and probed with a radiolabeled ORF0 fragment or a radiolabeled rubisco cDNA fragment. This cDNA fragment was synthesized against mRNA isolated from leaves of potato and a kind gift of J. P. H. Nap (DLO Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands).

For reverse transcription and subsequent PCR amplification, total plant RNA was isolated with the RNeasy Plant Total RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An oligonucleotide homologous to positions 70 to 89 of the PLRV genome (van der Wilk et al. 1989) was used as 5′ primer and an oligonucleotide complementary to the nucleotides 798 to 812 of the PLRV genome was used as downstream primer.

Analysis f genomic DNA in transgenic potato plants.

For Southern analysis, genomic DNA was isolated from the plant material as described by Dellaporte et al. (1983). The genomic DNA was digested with restriction enzymes, separated on agarose gel, and subsequently transferred to a blotting membrane (Hybond-N) (Sambrook et al. 1989). The blots were hybridized with a radiolabeled ORF0 probe or rubisco probe.

PCR on genomic DNA was carried out with oligonucleotides identical to a sequence in the CaMV 35S-promoter (92 nucleotides upstream of the start codon of ORF0 in the binary construct) and complementary to the 3′ end of the ORF0.

Analysis of the PLRV p28 protein in transgenic potato plants.

Transgenic and PLRV-infected plants were analyzed for p28 protein expression with ELISA and Western blot procedures. To analyze the presence of accumulated p28, antisera were used that had been raised against two synthetic peptides (ELISA) and raised against a p28-glutathione-S-transferase (GST) fusion protein produced in *Escherichia coli* (Western blot). The amino acid sequence of p28 was analyzed with the help of the GCG computer program Peptidestructure (Jameson and Wolf 1988; Devereux et al. 1984). Two domains with a high antigenic index were identified at amino acid positions 64 to 83 and 205 to 224, respectively. Two synthetic peptides identical to the identified domains were synthesized, with amino acid sequences CKRGRISTSGLQLPRHLHYE (SYN1) and ARLYNQLDLQGRAKSFRALT (SYN2), respectively. The synthetic peptides were covalently conjugated to the carrier protein keyhole limpet haemocyanin (KLH; M_R 4.5×10^4 to 1.3×10^7) utilizing 1-ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride (EDC) as a coupling reagent. Coupling reactions and subsequent purification of the conjugate were carried out with a commercially available kit (Imject Immunogen EDC Conjugation Kit, Pierce, Rockford, IL) according to the manufacturer's instructions. Rabbits were immunized by two subcutaneous injections at a 3-week-interval with approximately 100 µg of conjugate emulsified with equal volumes of Freund's incomplete adjuvant, 3 three weeks later by a subcutaneous booster injection with approximately 200 µg conjugated protein. One week after this injection, blood was taken from the rabbits and tested for immunogenic response in ELISA. Gamma-globulin fractions were purified from the serum according to Clark and Adams (1977).

To raise an antiserum suitable in Western blot procedures, the *Bam*HI-equipped ORF0 fragment was ligated in the bacterial expression vector pGEX-2T and the obtained construct (pGEXP28) was used to transform JM101 cells. Cells harboring the plasmid were grown to mid-log phase at 37°C, after which expression was induced by adding isopropyl-a-D-thiogalactoside (IPTG) to a final concentration of 1.0 mM to the culture. The cells were allowed to grow further for 5 h and subsequently harvested by centrifugation. To monitor expression of p28, the cells were resuspended in Laemmli buffer (Laemmli 1970) and boiled for 10 min. The samples were loaded onto a 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel and subjected to electrophoresis. After electrophoresis the proteins were visualized either with Coomassie brilliant blue or by silver staining. Denatured p28 was isolated from the SDS polyacrylamide gel with a Prep-Cell electrophoresis apparatus (BioRad, Richmond, CA). Purified, denatured protein (5 µg) was emulsified in Freund's incomplete adjuvant (Difco, Detroit, MI) and injected four times into two mice at 3-week intervals. One week after each injection the mice were bled. Gamma-globulin fractions were isolated from the serum by ammonium sulfate precipitation (Clark and Adams 1977).

ELISA was performed as described (van den Heuvel and Peters 1989); transgenic plant tissues were ground in extraction buffer (0.2% ovalbumin, 2% polyvinylpyrrolidone, 0.05% Tween 20 in phosphate-buffered saline). Two hundred microliters of the suspension was used per well. ELISA plates were coated (0.5 µg/ml) with IgGs raised against synthetic peptides. IgGs conjugated to alkaline phosphatase were used to detect bound p28. Western blot was carried out with a buffered transfer system. Leaf material or purified protein fractions were ground in Laemmli buffer (Laemmli 1970), boiled for 10 min, and loaded onto a 12.5% SDS polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose and probed with antiserum.

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